University of Zurich[UZH]

Zurich Open Repository and Archive
University of Zurich
Main Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2010

Die Rolle von Tissue Factor im Akuten Koronarsyndrom: Molekulare Mechanismen und Klinische Implikationen

Steffel, Jan

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-61265
Habilitation

Originally published at:
Steffel, Jan. Die Rolle von Tissue Factor im Akuten Koronarsyndrom: Molekulare Mechanismen und Klinische Implikationen. 2010, University of Zurich, Faculty of Medicine.

Kumulative Habilitationsschrift
zur Erlangung der Venia Legendi der Universität Zürich

Die Rolle von Tissue Factor im Akuten Koronarsyndrom
Molekulare Mechanismen und Klinische Implikationen

vorgelegt von Dr. Jan Steffel
von Bonn, Deutschland

Zürich, den 01.09.2010

Inhaltsverzeichnis

Vorwort 3
Behandelte Originalarbeiten 3
Zusammenfassung 4
Referenzen 11
Steffel J., Akhmedov A., Greutert H., Luscher T.F., Tanner F.C.: **Histamine induces tissue factor expression: implications for acute coronary syndromes.**
Circulation. 2005 Jul 19;112(3):341-9. 12
Steffel J., Arnet C., Akhmedov A., Iseli S.M., Lüscher T.F., Tanner F.C.: **Histamine differentially interacts with TNF-alpha and thrombin in endothelial tissue factor** induction – Role of c-Jun terminal NH kinase.
Journal of Thrombosis and Haemostasis. 2006 Nov;4(11):2452-60. 21
Steffel J.*, Latini R.A.*, Akhmedov A., Zimmerman D., Zimmerling P., Luscher T.F., Tanner F.C.: **Rapamycin, but not FK-506, increases endothelial tissue factor expression - Implications for drug-eluting stent design**. * Equal contribution.
Circulation. 2005 Sep 27;112(13):2002-11. 30
Stähli B.E., Camici G.G., Steffel J., Akhmedov A., Shojaati K., Gramer M., Luscher T.F., Tanner F.C.: **Paclitaxel enhances thrombin-induced endothelial tissue factor expression via c-Jun terminal NH2 kinase activation.**
Circulation Research. 2006 Jul 21;99(2):149-55. 40
Steffel J., Lüscher T.F., Tanner F.C.: **Tissue Factor in Cardiovascular Diseases.**
Circulation. 2006 Feb 7;113(5):722-31 47
Lüscher T.F., Steffel J., Eberli F.R., Joner M., Nakazawa G., Tanner F.C., Virmani R.: **Drug-eluting stents and coronary thrombosis: biological mechanisms and clinical implications.**
Circulation. 2007;115(8):1051-8. 57

Vorwort

Die vorliegende Habilitationsarbeit umfasst Arbeiten, welche ich im Laufe meiner kontinuierlichen Forschungsarbeiten auf dem Gebiet der molekularen Regulation von Tissue Factor während der letzten Jahre angefertigt habe.

Die nachfolgend aufgeführten 4 Originalarbeiten werden als kumulative Habilitationsschrift eingereicht. In der vorstehenden Synopsis sollen diese in den Kontext vergangener und aktueller Forschung auf diesem Gebiet gestellt, sowie ihre Bedeutung für das aktuelle Verständnis und für weitere klinische und grundlagenwissenschaftliche Arbeiten dargelegt werden.

Originalarbeiten

Steffel J., Akhmedov A., Greutert H., Luscher T.F., Tanner F.C.: Histamine induces tissue factor expression: implications for acute coronary syndromes.
Circulation. 2005 Jul 19;112(3):341-9.

Steffel J., Arnet C., Akhmedov A., Iseli S.M., Lüscher T.F., Tanner F.C.: Histamine differentially interacts with TNF-alpha and thrombin in endothelial tissue factor induction – Role of c-Jun terminal NH kinase.
Journal of Thrombosis and Haemostasis. 2006 Nov;4(11):2452-60.

Steffel J.*, Latini R.A.*, Akhmedov A., Zimmerman D., Zimmerling P., Luscher T.F., Tanner F.C.: Rapamycin, but not FK-506, increases endothelial tissue factor expression - Implications for drug-eluting stent design. * Equal contribution.
Circulation. 2005 Sep 27;112(13):2002-11.

Stähli B.E., Camici G.G., Steffel J., Akhmedov A., Shojaati K., Gramer M., Luscher T.F., Tanner F.C.: Paclitaxel enhances thrombin-induced endothelial tissue factor expression via c-Jun terminal NH2 kinase activation.
Circulation Research. 2006 Jul 21;99(2):149-55.

Summary

Introduction

Tissue factor (TF), formerly known as thromboplastin, is the key initiator of the coagulation cascade.[1] The latter is triggered as soon as TF comes into contact with circulating activated factor VII (VIIa) resulting in formation of the TF-FVIIa complex (Fig. 1). The TF-VIIa complex then activates factor IX, which in turn activates factor X. In an important step of the coagulation cascade, the complex of factor Xa, calcium, and Factor Va catalyzes the conversion of prothrombin to thrombin, leading to fibrin formation, platelet activation, and, ultimately, generation of a thrombus (Fig 1).

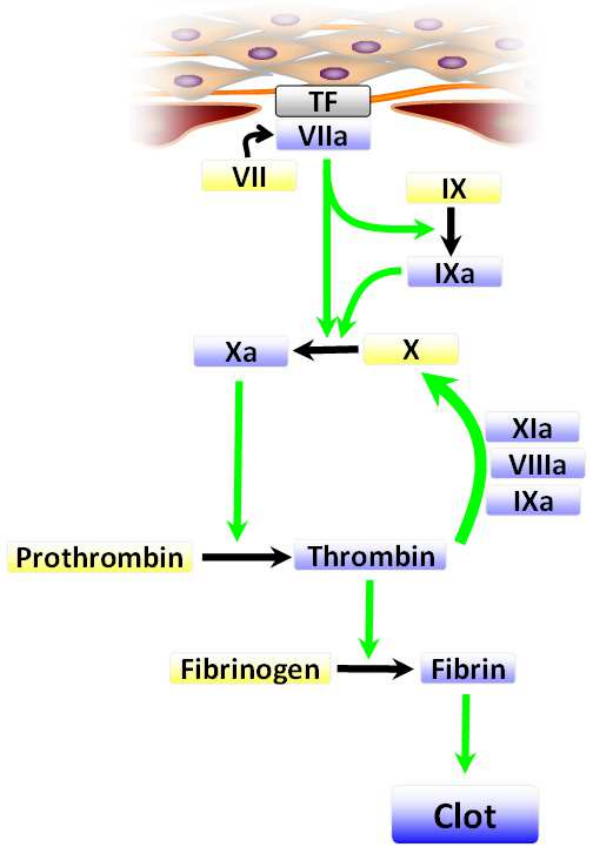


Fig. 1: Tissue factor (TF) – key initiator of the coagulation cascade. Modified from: Steffel et al., Circulation 2006.

Over the last decade TF, has been recognized to be involved in the pathogenesis of a plethora of cardiovascular diseases. Moreover, our understanding of the molecular regulation of TF expression in vascular cells has profoundly improved. The following summary will focus on the role of TF in acute coronary syndromes and in the pathogenesis of stent thrombosis.

Role of tissue factor in acute coronary syndromes

In acute coronary syndromes (ACS), an occlusive thrombus leads to complete cessation of epicardial blood flow, eventually resulting in the clinical sequelae of a myocardial infarction (Fig. 2). Various lines of evidence indicate that TF is importantly involved in the pathogenesis of ACS. Indeed, TF can be detected in various different cell types in atheromatous plaques including endothelial and vascular smooth muscle cells, as well as in the necrotic plaque core.[1] Moreover, elevated levels of TF have been observed in atherectomy specimens as well as in plasma of patients with unstable angina.

In addition to macrophages and T-lymphocytes, increasing evidence has been accumulating that mast cells may also be involved in the development of atherosclerotic lesions as well as in the pathogenesis of ACS. Indeed, mast cell activation occurs as an inflammatory event in atherogenesis and may lead to plaque instability.[2] Furthermore, mast

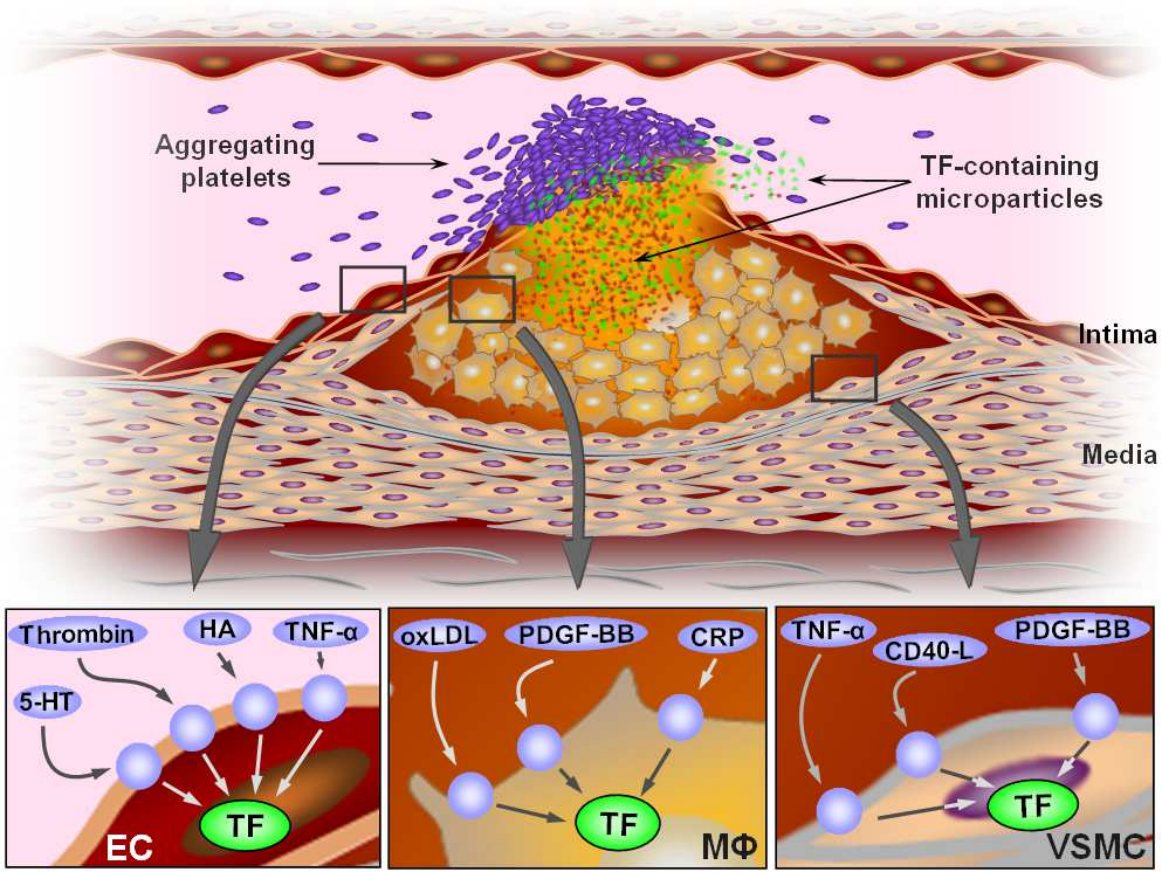


Fig. 2: Tissue factor (TF) in atherosclerotic plaque rupture.
5-HT – Serotonine; CD40-L – CD40-ligand; CRP – C-reactive protein; EC – Endothelial cell; HA – Histamine; ox LDL – Oxidized low-density lipoprotein; MΦ – Macrophage; PDGF-BB – Platelet derived growth factor BB; TNF-α – Tumor necrosis factor alpha; VSMC – Vascular smooth muscle cell. From: *Steffel et al.*, Circulation 2006.

cells are present at the site of vasospasm in patients with variant angina, suggestive of an involvement of histamine in coronary artery spasm.[3] Importantly, previous studies have indicated that coronary artery spasm may in itself lead to thrombus formation and vascular occlusion. The exact link between thrombosis and vasospasm, however, remained elusive. We were able to demonstrate that histamine, a biogenic amine mainly released by mast cells, potently induces TF in endothelial cells.[4] This induction occurred at the transcriptional level, and was mediated via activation of the MAP kinases p38, extracellular

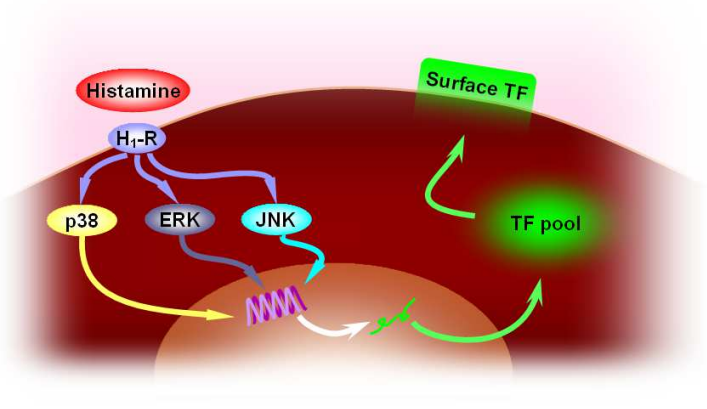
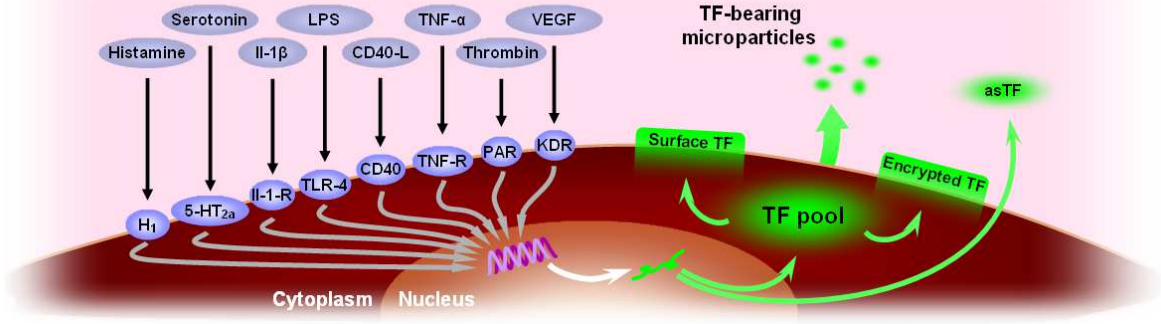


Fig. 3: Mediators of histamine-induced tissue factor (TF) induction. See text for details.

signal-regulated kinase (ERK), and c-jun terminal NH_2 kinase (JNK, Fig. 3). In contrast to these MAP kinases, the phosphatidylinositol 3 (PI-3) -kinase pathway is constitutively active in endothelial cells, leading to a suppression of basal endothelial TF expression. Stimulation with histamine led to an inhibition of the PI3 kinase pathway, and hence to an increase in TF expression. On the receptor level, histamine-induced TF expression was completely blocked by different H_1-receptor antagonists, while H_2-receptor blockade had no effect. The latter is in good accordance with numerous previous studies, indicating that most deleterious vascular effects of histamine including vasospasm and intimal thickening after acute arterial injury are mediated via the H_1-receptor.

In the inflammatory environment of atherogenesis, however, histamine is by far not the only active pathogen; indeed, various cytokines and mediators accumulate in the



<u>Fig. 4:</u> Mediators of tissue factor (TF) induction.
$5\text{-}HT_{2a}$ – 5-hydroxytryptamine$_{2a}$ receptor; asTF - Alternatively spliced tissue factor. CD40-L – CD40-ligand; H_1 – Histamine H_1-receptor; IL-1 – Interleukin-1; IL1-R – Interleukin-1 receptor; KDR – VEGF receptor-2; LPS – Lipopolysaccharide; PAR – Protease-activated receptor; TLR-4 – Toll-like receptor 4; TNF-α – Tumor necrosis factor alpha; VEGF – Vascular endothelial growth factor. From: *Steffel et al.*, Circulation 2006.

developing atherosclerotic plaque and are released upon plaque rupture (Fig. 3 and Fig. 4). In order to better understand the role of histamine in thrombus formation, we investigated the interplay between histamine and two other important mediators of thrombus formation, TNF-α and thrombin. While both of these agents are renown for their potent TF inducing capacity, histamine resulted in a substantial potentiation of TNF-α-induced TF expression, whereas thrombin-induced TF expression was only slightly enhanced.[5] We were able to show that this differential augmentation occurred due to differential activation of JNK, resulting in pronounced synergy between histamine and TNF-α at the subcellular level. These findings are in line with other observations indicating a remarkable synergy of histamine and TNF-α on vascular cells, including the induction of intracellular adhesion molecule 1 and E-selectin.[6] Similar to our previous observations, the augmentation in TF induction by histamine was completely abolished by H_1-receptor antagonist pretreatment. This study hence adds to the

evidence that histamine and TNF-α effectively interact in endothelial activation as well as in vascular inflammation.

Taken together, these two studies demonstrate that histamine may be importantly involved in thrombus formation in acute coronary syndromes, both by directly inducing TF as well as by potentiating the induction of TF by other mediators. Indeed, histamine-induced TF expression may represent an important link between vasospasm and thrombosis. Since this effect is completely abolished by H_1-receptor antagonists, our results may furthermore have interesting therapeutic implications indicating a potential role for H_1-receptor blockers in the treatment of atherosclerotic vascular diseases.

Role of tissue factor in stent thrombosis

Once thrombotic vessel occlusion occurs in ACS, percutaneous coronary intervention (PCI) and stenting of the culprit lesion is the preferred invasive treatment.[7] Drug-eluting stents (DES) release pharmacological substances into the vessel wall after deployment which inhibit vascular smooth muscle cells migration and proliferation, i.e. key elements in the pathogenesis of restenosis. Several clinical trials have shown that DES are superior to

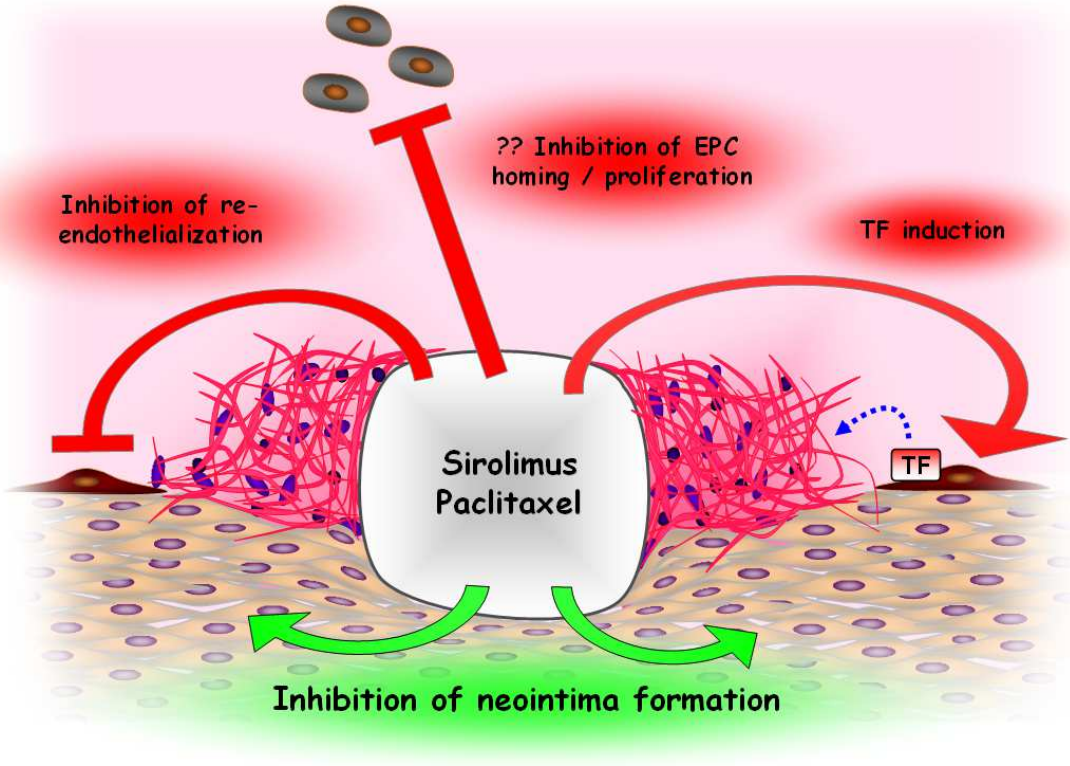


Fig. 5: Potential mechanisms of thrombogenesis in first-generation drug-eluting stents.
EPC – Endothelial progenitor cells; TF – Tissue factor. From: *Lüscher, Steffel et al.,* Circulation 2007.

uncoated bare-metal stents (BMS), mainly based on a decrease in restenosis rates and major adverse cardiac events. Despite reduced restenosis rates, however, the occurrence of stent

thromboses have not decreased with DES as compared to BMS, and may even be higher in the former under certain circumstances.[8] The reason for the discrepancy between reduced restenosis rates and possibly enhanced thrombosis rates with first-generation DES as compared to BMS is not entirely unraveled yet, but likely results from the different mechanism underlying these two entities. While in the former, migration and proliferation of vascular smooth muscle cells in the neointimal lead to gradual re-occlusion of the target lesion, stent thrombosis occurs as a result of acute activation of the coagulation cascade and sudden thrombotic stent occlusion. As a result, the clinical sequelae differ considerably, with increasing, "typical" angina pectoris resulting from restenosis as compared to a sudden, acute myocardial infarction resulting from stent thrombosis. As a result, stent thrombosis remains one of the most feared complications in interventional cardiology owing to its high morbidity and mortality.

Several factors are associated with an increased risk of stent thrombosis: These include factors associated with the procedure itself (stent malapposition and/or underexpansion, stent length, number of implanted stents, and dissections), patient and lesion characteristics, stent design as well as, importantly, premature cessation of antiplatelet drugs. Moreover, drugs loaded on DES may contribute significantly to the thrombogenicity of the stent by several mechanisms (Fig. 5). Importantly, these drugs are designed to inhibit migration and proliferation of vascular smooth muscle cells, thereby inhibiting an important process contributing to in-stent restenosis as outlined above. By the same intracellular mechanisms, however, they may equally inhibit endothelial cell migration and proliferation, and hence impede stent re-endothelialization. With the stent (i.e., a foreign body) exposed to the blood stream without a "protective" endothelial cover, activation of the coagulation cascade may readily occur. Furthermore, inhibition of endothelial progenitor cell homing to the site of stent implantation by the drugs loaded on DES may reduce endothelial cell coverage and subsequently increase stent thrombogenicity even further.

Above and beyond inhibition of re-endothelialization, however, drugs loaded on DES may have other effects, which may contribute to DES thrombogenicity. In first-generation DES, rapamycin (sirolimus, a macrocyclic lacton), FK-506 (tacrolimus, a macrolide immunosuppressant) and paclitaxel (a microtubule-stabilizing drug) are frequently used for DES coating. We were able to demonstrate that both rapamycin and paclitaxel induce endothelial TF expression and surface activity, and potentiate TF induction by other mediators (i.e. thrombin and TNF-α).[9, 10] The subcellular mechanisms, however, were distinctly different for the two drugs (Fig. 6). Rapamycin exerts most of its biological effects by binding to its intracellular receptor, FKBP-12, which in turn inhibits the activity of mTOR (mammalian target of rapamycin). The latter is a downstream target of the PI-3 kinase, which, as previously mentioned, exerts an inhibitory effect on endothelial TF expression. By inhibiting mTOR, rapamycin induced TF via a "disinhibition" of the PI-3 kinase pathway; in contrast, phosphorylation of the MAP kinases, which are equally important regulators of TF, remained

unaffected. Importantly, rapamycin-induced TF expression could be entirely blunted by FK-506, which competitively binds to the same intracellular receptor (i.e., FKBP-12), but subsequently inhibits the phosphatase calcineurin without affecting mTOR. Consequently, FK-506 itself had no significant effect on TF expression. In subsequent in-vivo studies, we were able to confirm this hypothesis and demonstrate that treatment with rapamycin accelerates carotid artery vessel occlusion.[11]

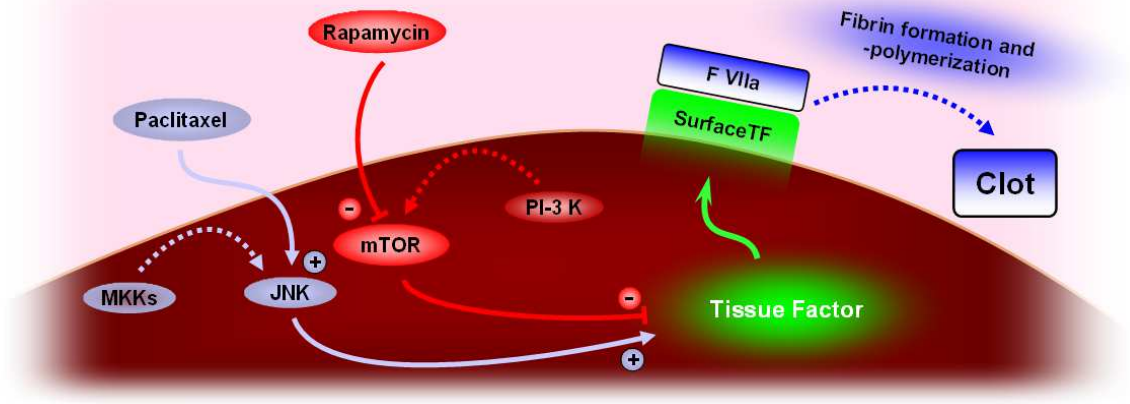


Fig. 6: Mechanism of rapamycin- and paclitaxel induced tissue factor (TF) expression.
MKK – Map kinase kinases; JNK – C-jun terminal NH_2 kinase; PI-3K – phosphatidylinositol-3 kinase; mTOR – Mammalian target of rapamycin. From: *Lüscher, Steffel et al.,* Circulation 2007.

In contrast to rapamycin, the mechanism underlying TF-induction by paclitaxel was distinctly different. Indeed, the latter had no effect on the PI-3 kinase pathway at all, but instead potently and selectively activated c-Jun terminal NH2 kinase (JNK) while leaving phosphorylation of the MAP kinases p38 and ERK unaffected. Docetaxel, a related microtubule-stabilizing agent, exerted a similar effect on JNK activation and TF expression as paclitaxel, indicating that TF induction by paclitaxel depends on stabilization of microtubule bundles rather than being a substance-specific effect.

In summary, both rapamycin and paclitaxel increase endothelial TF expression and surface activity, which may favor the development of thrombus formation after deployment of drug-eluting stents. Both rapamycin and paclitaxel potentiate TF induction by other inflammatory or pro-thrombotic mediators, indicating that this effect may be particularly pronounced in the inflammatory environment of an acute coronary syndrome. As rapamycin-induced TF expression was blocked by FK-506, these data may have interesting implications for the design of future drug-eluting stents.

Conclusion

Tissue Factor, the main initiator of the coagulation cascade, is importantly involved in the pathogenesis of acute coronary syndromes. We were able to show that histamine induces TF in endothelial as well as in vascular smooth muscle cells, which is even further potentiated by interaction with other inflammatory mediators such as TNF-α. Furthermore, we demonstrated that rapamycin and paclitaxel, both substances used for drug-eluting stent coating, induce endothelial TF expression, which may favor the development of stent thrombosis, especially in the inflammatory setting of acute coronary syndromes. These findings enhance our knowledge of TF, both on the subcellular level and from a clinical point of view, and may have important therapeutic implications in the medical and invasive treatment of atherosclerotic vascular diseases.

References

1. Steffel J, Luscher TF, Tanner FC. Tissue factor in cardiovascular diseases: molecular mechanisms and clinical implications. *Circulation.* 2006;113:722-731.

2. Libby P. Inflammation in atherosclerosis. *Nature.* 2002;420:868-874.

3. Shimokawa H, Tomoike H, Nabeyama S, Yamamoto H, Araki H, Nakamura M, Ishii Y, Tanaka K. Coronary artery spasm induced in atherosclerotic miniature swine. *Science.* 1983;221:560-562.

4. Steffel J, Akhmedov A, Greutert H, Luscher TF, Tanner FC. Histamine induces tissue factor expression: implications for acute coronary syndromes. *Circulation.* 2005;112:341-349.

5. Steffel J, Arnet C, Akhmedov A, Iseli SM, Luscher TF, Tanner FC. Histamine differentially interacts with TNF-alpha and thrombin in endothelial tissue factor induction Role of c-Jun terminal NH kinase. *J Thromb Haemost.* 2006.

6. Miki I, Kusano A, Ohta S, Hanai N, Otoshi M, Masaki S, Sato S, Ohmori K. Histamine enhanced the TNF-alpha-induced expression of E-selectin and ICAM-1 on vascular endothelial cells. *Cell Immunol.* 1996;171:285-288.

7. Grines CL, Cox DA, Stone GW, Garcia E, Mattos LA, Giambartolomei A, Brodie BR, Madonna O, Eijgelshoven M, Lansky AJ, O'Neill WW, Morice MC. Coronary angioplasty with or without stent implantation for acute myocardial infarction. Stent Primary Angioplasty in Myocardial Infarction Study Group. *N Engl J Med.* 1999;341:1949-1956.

8. Luscher TF, Steffel J, Eberli FR, Joner M, Nakazawa G, Tanner FC, Virmani R. Drug-eluting stent and coronary thrombosis: biological mechanisms and clinical implications. *Circulation.* 2007;115:1051-1058.

9. Steffel J, Latini RA, Akhmedov A, Zimmermann D, Zimmerling P, Luscher TF, Tanner FC. Rapamycin, but not FK-506, increases endothelial tissue factor expression: implications for drug-eluting stent design. *Circulation.* 2005;112:2002-2011.

10. Stahli BE, Camici GG, Steffel J, Akhmedov A, Shojaati K, Graber M, Luscher TF, Tanner FC. Paclitaxel enhances thrombin-induced endothelial tissue factor expression via c-Jun terminal NH2 kinase activation. *Circ Res.* 2006;99:149-155.

11. Camici GG, Steffel J, Amanovic I, Breitenstein A, Baldinger J, Keller S, Luscher TF, Tanner FC. Rapamycin promotes arterial thrombosis in vivo: implications for everolimus and zotarolimus eluting stents. *Eur Heart J.* 2010; 2010 Jan;31(2):236-42.

Histamine Induces Tissue Factor Expression
Implications for Acute Coronary Syndromes

Jan Steffel, MD; Alexander Akhmedov, PhD; Helen Greutert; Thomas F. Lüscher, MD; Felix C. Tanner, MD

Background—Histamine can induce coronary vasospasm, leading to variant angina and acute myocardial infarction. However, the role of histamine in thrombus formation is ill defined. Hence, this study investigates whether histamine induces tissue factor (TF) expression in vascular cells.

Methods and Results—Histamine (10^{-8} to 10^{-5} mol/L) induced TF expression in a concentration-dependent manner in human aortic endothelial and vascular smooth muscle cells, whereas TF pathway inhibitor expression remained unaffected. RT-PCR and Northern blotting revealed that histamine stimulated TF mRNA transcription, peaking at 1 hour. Protein expression increased 18-fold ($P<0.02$) with a maximum at 5 hours, which was paralleled by a 4-fold augmentation in surface activity ($P<0.01$). These effects were completely prevented by pretreatment with the H_1 receptor antagonists mepyramine ($P<0.0001$), chlorpheniramine, and diphenhydramine but not the H_2 receptor antagonist cimetidine (P=NS). Histamine induced a time-dependent, H_1 receptor–mediated activation of p38 MAP kinase (p38), p44/42 MAP kinase (ERK), and c-jun terminal NH_2 kinase (JNK). Blocking of p38, ERK, or JNK with SB203580 ($P<0.0001$), PD98059 ($P<0.0001$), or SP600125 ($P<0.0001$), respectively, impaired histamine-induced TF expression in a concentration-dependent manner. In contrast, histamine-stimulated TF expression was increased by phosphatidylinositol 3-kinase inhibition with LY294002 or wortmannin, whereas it was not affected by Rho-kinase inhibition with Y-27632 or hydroxyfasudil.

Conclusions—Histamine induces expression of TF, but not TF pathway inhibitor, in vascular cells via activation of the H_1, but not H_2, receptor. This effect is mediated by the MAP kinases p38, ERK, and JNK. This observation may open novel perspectives in the treatment of variant angina and acute coronary syndromes. (***Circulation*. 2005;112:341-349.**)

Key Words: coagulation ■ coronary disease ■ endothelium ■ signal transduction ■ thrombosis

Histamine is a biogenic amine that is released by mast cells, endothelial cells, and aggregating platelets.[1–3] Mast cell activation is involved in atherogenesis and coronary artery disease.[4,5] Indeed, an elevated number of mast cells has been observed in coronary atherectomy specimens from culprit lesions, eliciting acute coronary syndromes.[6] Furthermore, an increased number of mast cells at the site of plaque erosion or rupture was found in patients who died of myocardial infarction.[7] Thus, mast cell activation occurs as an inflammatory event in atherogenesis and may be involved in plaque instability.[8,9] These observations suggest a role for histamine in the pathogenesis of acute coronary syndromes. Moreover, mast cells have been detected at the site of vasospasm in patients with variant angina, indicating a role for histamine in coronary artery spasm.[10,11] The latter has been implicated in the pathogenesis of thrombus formation leading to vascular occlusion[12,13]; indeed, the combination of vasoconstriction and thrombosis plays a major role in intermittent coronary artery occlusion after thrombolysis.[14] Hence, a dynamic interaction of atherosclerosis, vasospasm, and thrombus formation may precede vascular occlusion in acute coronary syndromes.

Tissue factor (TF), a 263-residue membrane-bound glycoprotein, is a key enzyme in the activation of coagulation; it binds activated factor VII (FVIIa), which in turn activates factor X (FX), ultimately leading to thrombin formation. Because TF pathway inhibitor (TFPI) is the direct physiological inhibitor of the TF/FVIIA complex, it can modulate the effect of TF on initiation of coagulation. Initiation of coagulation plays a major role in the pathogenesis of acute coronary syndromes. Accordingly, TF can be detected in various cell types in atheromatous plaques, including endothelial and vascular smooth muscle cells.[15] Furthermore, elevated levels of TF antigen and activity have been detected in plasma and atherectomy specimens of

Received August 20, 2004; revision received November 28, 2004; second revision received April 5, 2005; accepted April 11, 2005.

From Cardiovascular Research, Physiology Institute, University of Zürich (J.S., A.A., H.G., T.F.L., F.C.T.), Center for Integrative Human Physiology, University of Zürich (J.S., A.A., H.G., T.F.L., F.C.T.), and Cardiology, Cardiovascular Center, University Hospital Zürich (J.S., T.F.L., F.C.T.), Zürich, Switzerland.

Guest Editor for this article was Roberto Bolli, MD.

Correspondence to Felix C. Tanner, MD, Cardiovascular Research, Physiology Institute, University of Zurich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. E-mail felix.tanner@access.unizh.ch

© 2005 American Heart Association, Inc.

Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/CIRCULATIONAHA.105.553735**

patients with unstable angina.[15] Thus, TF is involved in the initiation and propagation of acute coronary syndromes.

In the present study, we show that histamine induces TF expression and activity in human aortic endothelial cells (HAECs) and human vascular smooth muscle cells (HAVSMCs). This effect, mediated through activation of the MAP kinase pathway, is completely abolished by H_1 receptor antagonists.

Methods

Cell Culture

HAECs and HAVSMCs were cultured as described.[16] Cells were grown to confluence in 6-cm culture dishes and rendered quiescent for 24 hours before stimulation with histamine (Sigma). Mepyramine, chlorpheniramine, diphenhydramine, and cimetidine (all from Sigma) were added to the dishes 30 minutes before stimulation. Y-27632, hydroxyfasudil, wortmannin, SB203580 (all from Sigma), LY294002, PD98059 (both from Cell Signaling), and SP600125 (Calbiochem) were added 60 minutes before stimulation. To assess cytotoxicity, a colorimetric assay for detection of lactate dehydrogenase was used according to the manufacturer's recommendations (Roche). A commercially available limulus amoebocyte lysate assay was used according to the manufacturer's recommendations (Cambrex) to assess for potential contamination with endotoxin. For some experiments, histamine and/or lipopolysaccharide (LPS) solutions were heated in a boiling water bath for 1 hour before being added to the cells.

Western Blot Analysis

Protein expression was determined by Western blot analysis. Cells were lysed in 50 mmol/L TRIS buffer as described.[16,17] Then, 40-μg samples were loaded and separated by 10% SDS-PAGE. Proteins were transferred to a PVDF membrane (Millipore) by semidry transfer. Equal loading was confirmed by Ponceau S staining. Antibodies to human TF and TFPI (both from American Diagnostica) were used at 1:2000 and 1:1000 dilution, respectively; antibodies against phosphorylated p38 MAP kinase (p38), phosphorylated p44/42 MAP kinase (ERK), and phosphorylated c-jun terminal NH_2 kinase (JNK; all from Cell Signaling) were used at 1:1000, 1:5000, and 1:1000 dilution, respectively. Antibodies against total p38, total ERK, and total JNK (all from Cell Signaling) were used at 1:2000, 1:10 000, and 1:1000 dilution, respectively. Blots were normalized to α-tubulin expression (1:10 000 dilution; Sigma).

RT-PCR and Northern Blot Analysis

Total RNA was extracted from HAECs (1.5×10^6 cells) with 1 mL TRIzol Reagent (Invitrogen) according to the manufacturer's recommendations. Conversion of total cellular RNA to cDNA was carried out with Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Amersham Biosciences) in a final volume of 35 μL using 4 μg of RNA. The total cDNA pool obtained served as a template for subsequent PCR amplification with TF (F3) -specific primers (508 to 529 bp, 892 to 913 of F3 cDNA; NCBI No. NM 001993).[18] The PCR conditions were as follows: 25 μL final reaction containing 2.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 10 μmol/L of each primer, 2.5 μL of dimethyl sulfoxide, 0.5 U of TaKaRa Taq DNA polymerase (TaKaRa Biomedicals), and 2 μL of cDNA template by using the following cycling parameters: 94° for 3 minutes for 1 cycle, 94° for 1 minute, 60° for 30 seconds, 65° for 45 seconds, for a total of 35 cycles, followed by a final elongation step at 65° for 5 minutes. S15 primers served as loading controls for PCR. Products were separated by electrophoresis on a 1% agarose gel and visualized with ethidium bromide.

Total RNA (15 to 20 μg) per sample was analyzed by Northern blotting. A 405-bp human TF cDNA (508 to 913 bp) fragment was labeled with [α-^{32}P]dCTP (Amersham Biosciences) using the Prime-It Kit (Stratagene) according to the manufacturer's recommendations. Afterward, the membrane was stripped and rehybridized with a control probe against the housekeeping gene human β-actin (Clontech). Bands were visualized by autoradiography.

TF Surface Activity

TF surface activity was analyzed with a colorimetric assay (American Diagnostica) according to the manufacturer's recommendations with some modifications as described.[19] Cells were grown in 6-well plates; after stimulation, cells were washed twice with PBS, followed by incubation with human FVIIa and FX at 37°, allowing the formation of a TF/FVIIa complex at the cell surface. The TF/FVIIa complex converted human FX to activated FX, which was measured by its ability to metabolize a chromogenic substrate. Lipidated human TF was used as a positive control to confirm that the results obtained were in the linear range of detection (data not shown).

Statistical Analysis

Data are presented as mean±SEM. An unpaired Student t test was applied to compare 2 groups; ANOVA with Bonferroni's correction was used for $\geq$3 groups. A value of $P<0.05$ was considered significant.

Results

Histamine Stimulates TF Expression and Activity in HAECs and HAVSMCs

Stimulation of HAECs with histamine (10^{-8} to 10^{-5} mol/L) led to a concentration- and time-dependent induction of TF expression up to 18-fold the basal level; a maximal effect was observed with 10^{-5} mol/L histamine (Figure 1A) and after 5 hours (Figure 1B). Under such conditions, the level of TF expression corresponded to more than one third of that observed with 10 ng/mL tumor necrosis factor (TNF)-α, a known potent inducer of TF expression in vascular cells (Figure 1D, left). Histamine also enhanced TF expression in HAVSMCs; the maximal increase was ≈3-fold compared with control conditions (Figure 1C), again corresponding to more than one third of the effect of 10 ng/mL TNF-α (Figure 1D, right).

TF expression is very sensitive to LPS. Therefore, a limulus amoebocyte lysate assay was performed that did not detect endotoxin in water or histamine samples (detection limit of the test, 0.125 EU/mL; n=3; data not shown). Moreover, the influence of heat treatment on histamine-induced TF expression was examined. Histamine is very heat stable,[20] whereas LPS is inactivated at least in part by boiling for 1 hour.[21] Boiling of histamine (10^{-5} mol/L) for 1 hour did not affect its ability to induce TF (P=NS; Figure 1E). In contrast, boiling of LPS (100 ng/mL) for 1 hour reduced its ability to induce TF expression by 81% ($P<0.0001$). LPS potentiated histamine-induced TF expression, whereas boiling of LPS (100 ng/mL) plus histamine for 1 hour resulted in a decrease in TF induction by 80% compared with unheated control (n=3; $P<0.0001$; Figure 1E). When a lower concentration of LPS (2 ng/mL) was added with histamine (10^{-5} mol/L), TF was reduced to the level observed after stimulation with histamine alone (n=3; data not shown), consistent with the observation that lower concentrations of LPS are more readily inactivated by heat treatment.[21] Thus, in contrast to histamine alone, boiling of LPS both alone and together with histamine resulted in a significantly reduced TF induction. These results demonstrate that no significant contamination of histamine solutions with LPS occurred and confirm

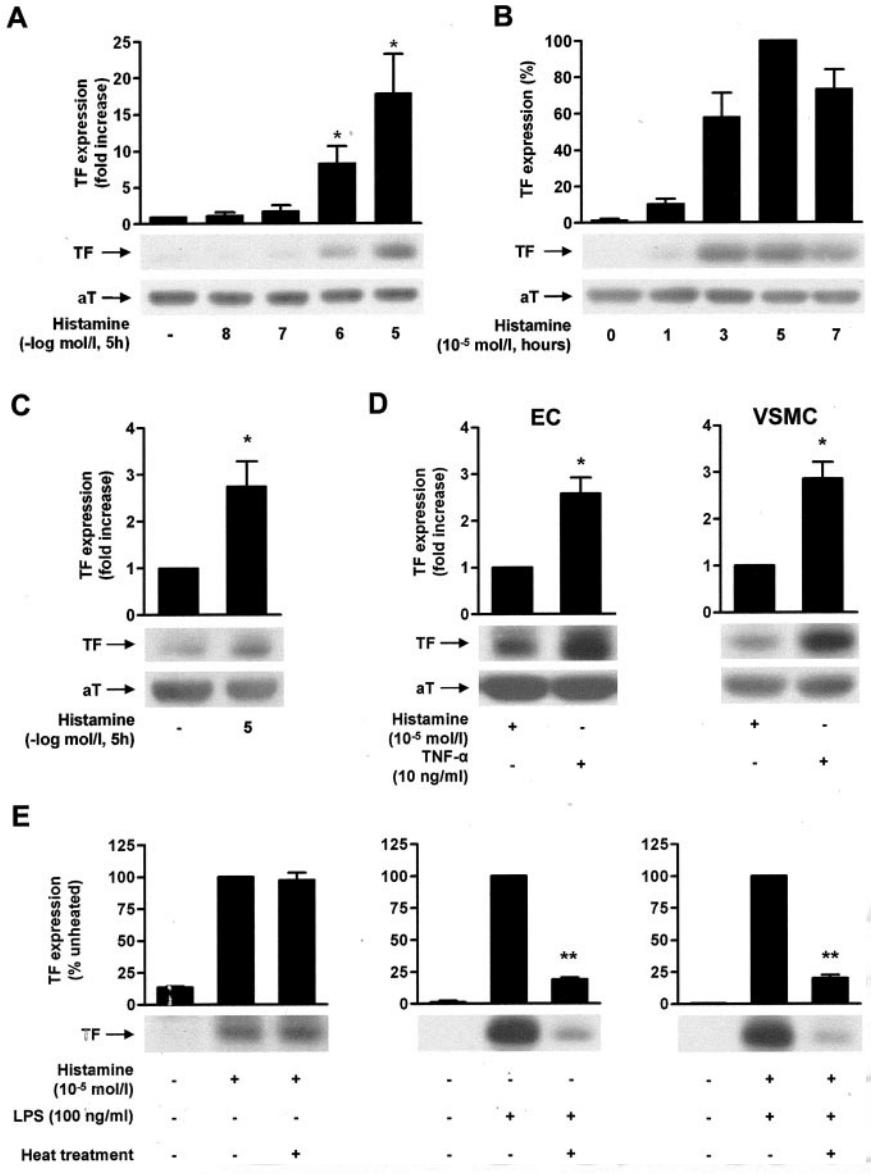


Figure 1. Histamine induces TF expression in HAECs and HAVSMCs. A, Concentration dependency of histamine-induced TF expression in HAECs. Values are given as fold increase over unstimulated control. *$P<0.02$ vs control. B, Time dependency of histamine-induced TF expression in HAECs. Values are given as percentage of TF expression at 5 hours. C, Histamine-induced TF expression in HAVSMCs. Values are given as fold increase over unstimulated control. *$P<0.02$ vs control. D, Comparison of histamine- and TNF-α–induced TF expression in HAECs (left) and HAVSMCs (right). *$P<0.01$ vs histamine. E, Heat treatment of histamine (left), LPS (middle), and histamine plus LPS (right). **$P<0.001$. Blots are representative of $\geq$3 different experiments and are normalized to α-tubulin (aT) expression.

that the observed induction of TF is due to the action of histamine.

RT-PCR (Figure 2A) and Northern blot analysis (Figure 2B) revealed that TF mRNA was induced in a time-dependent manner. Maximal upregulation was observed after 1 hour and then declined within 5 hours.

The increase in endothelial TF protein expression was reflected by a 4-fold increase in TF surface activity after stimulation with 10^{-5} mol/L histamine for 5 hours (Figure 3A). TF surface activity of HAVSMCs was increased to 1.4-fold the basal level under these conditions (Figure 3B). Omission of either FVIIa or FX was used as negative control for the specificity of the enzymatic reaction. No measurable increase in TF activity over background levels was observed under such conditions (n=3; P=NS; data not shown).

Histamine Does Not Affect Expression of TFPI

TFPI is the direct physiological inhibitor of the TF/FVIIa complex. Interestingly, stimulation with histamine did not affect TFPI expression in HAECs (Figure 4A). As a control, the same lysates were blotted for TF expression (Figure 4B), revealing the typical pattern of TF induction in response to histamine (Figure 1A).

Histamine-Induced TF Expression Is Mediated by Activation of the H_1 Receptor

Histamine is known to exert most of its actions on cells through the H_1 or H_2 receptor. To determine the receptor involved in histamine-induced TF expression, cells were pretreated with the H_1 receptor antagonists mepyramine (10^{-8} to 10^{-5} mol/L), chlorpheniramine (10^{-8} to 10^{-5} mol/L), or diphenhydramine (10^{-8} to 10^{-5} mol/L) or with the H_2 receptor antagonist cimetidine (10^{-7} to 10^{-5} mol/L). Preincubation with mepyramine completely abolished histamine-induced TF expression in HAECs (Figure 5A, top); the same effect was observed with chlorpheniramine and diphenhydramine (Figure 5A, middle and lower, respectively). In contrast, TF expression in response to histamine remained unaffected by

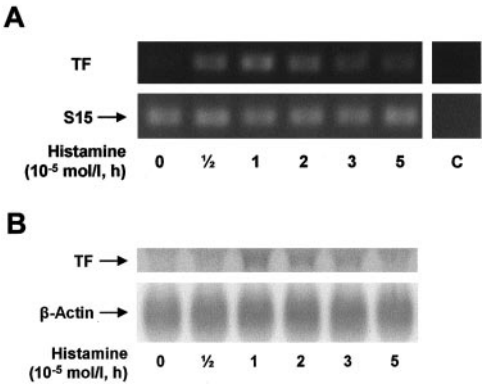


Figure 2. Histamine induces TF mRNA in HAECs. Time-dependent increase in TF mRNA expression is observed after stimulation of HAECs with histamine (10^{-5} mol/L). A, Representative RT-PCR; B, representative Northern blot analysis. S15 and human β-actin served as loading controls for RT-PCR and Northern blot analysis, respectively. Blots are representative of 3 independent experiments.

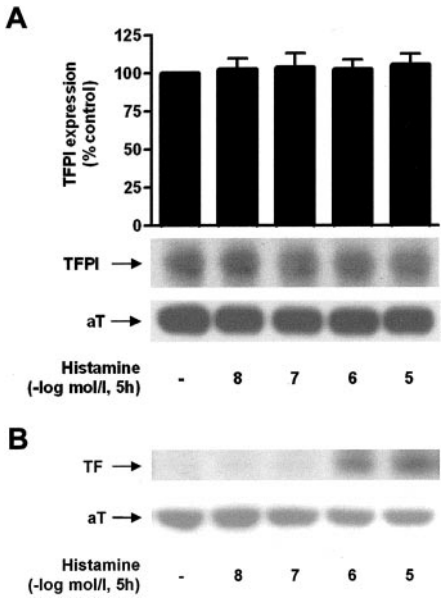


Figure 4. Histamine does not alter TFPI expression. A, Increasing concentrations of histamine do not alter TFPI expression in HAECs. Values are given as percent of unstimulated control. B, As control, samples from A are blotted for TF expression, revealing typical, concentration-dependent pattern of induction as demonstrated in A. Data are representative of ≥3 different experiments; all blots are normalized to α-tubulin (aT) expression.

preincubation with cimetidine (Figure 5B). Similarly, pretreatment of histamine-stimulated HAVSMCs with mepyramine (10^{-5} mol/L; Figure 5C) significantly reduced TF expression, whereas cimetidine (10^{-5} mol/L) did not (Figure 5D). Consistent with these observations, the histamine-induced increase in TF surface activity was inhibited by mepyramine (10^{-5} mol/L) in both HAECs (Figure 3A) and HAVSMCs (Figure 3B). Basal expression of TF was detected in HAVSMCs but not HAECs. A small increase in basal TF expression in HAVSMCs occurred after addition of both mepyramine (10^{-5} mol/L) and cimetidine (10^{-5} mol/L) in the absence of histamine; this effect was not observed in HAECs. Lactate dehydrogenase release did not reveal any cytotoxic effect of histamine, mepyramine, chlorpheniramine, diphenhydramine, or cimetidine (n=4; P=NS; data not shown).

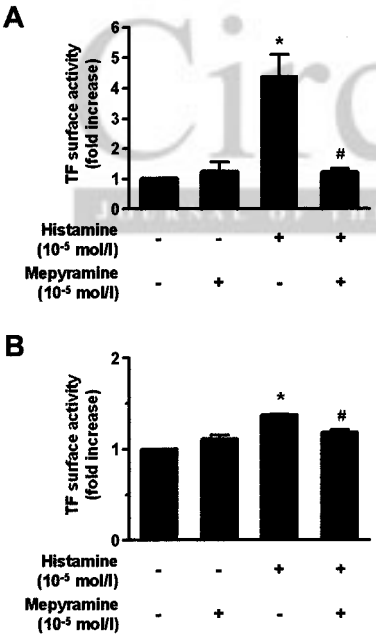


Figure 3. Histamine increases TF surface activity in HAECs and HAVSMCs. A, Histamine increases TF surface activity in HAECs. Increase in activity is suppressed by mepyramine. *P<0.01 vs control; #P<0.01 vs histamine. B, Histamine increases TF surface activity in HAVSMCs. Increase in activity is impaired by mepyramine. *P<0.0001 vs control; #P<0.01 vs histamine.

H_1-Receptor Activation Regulates TF Expression via the MAP Kinase Pathway

MAP kinase activation regulates TF expression in response to several mediators.[22,23] To assess whether histamine can activate MAP kinases, HAECs were examined at different time points after stimulation (Figure 6A). p38, ERK, and JNK were transiently activated after stimulation with histamine (10^{-5} mol/L). Maximal phosphorylation of p38 and ERK occurred after 5 minutes and returned to basal levels within 30 and 60 minutes, respectively. Maximal phosphorylation of JNK was observed after 15 minutes and declined to basal levels within 60 minutes. No change in total expression of p38, ERK, or JNK was observed at any time point. Blocking of the H_1 receptor with mepyramine (10^{-5} mol/L) completely abolished activation of p38, ERK, and JNK (Figure 6B). Again, total expression of the MAP kinases remained unchanged.

To analyze the involvement of MAP kinases in histamine-induced TF expression, we examined the effect of MAP kinase inhibitors on TF induction. SB203580 (10^{-6} to 10^{-5} mol/L), a specific inhibitor of p38, reduced histamine-induced TF expression in a concentration-dependent manner to 17% of control (Figure 7A). Similarly, preincubation with PD98059 (3×10^{-7} to 3×10^{-6} mol/L), a specific inhibitor of ERK phosphorylation, decreased TF expression in a

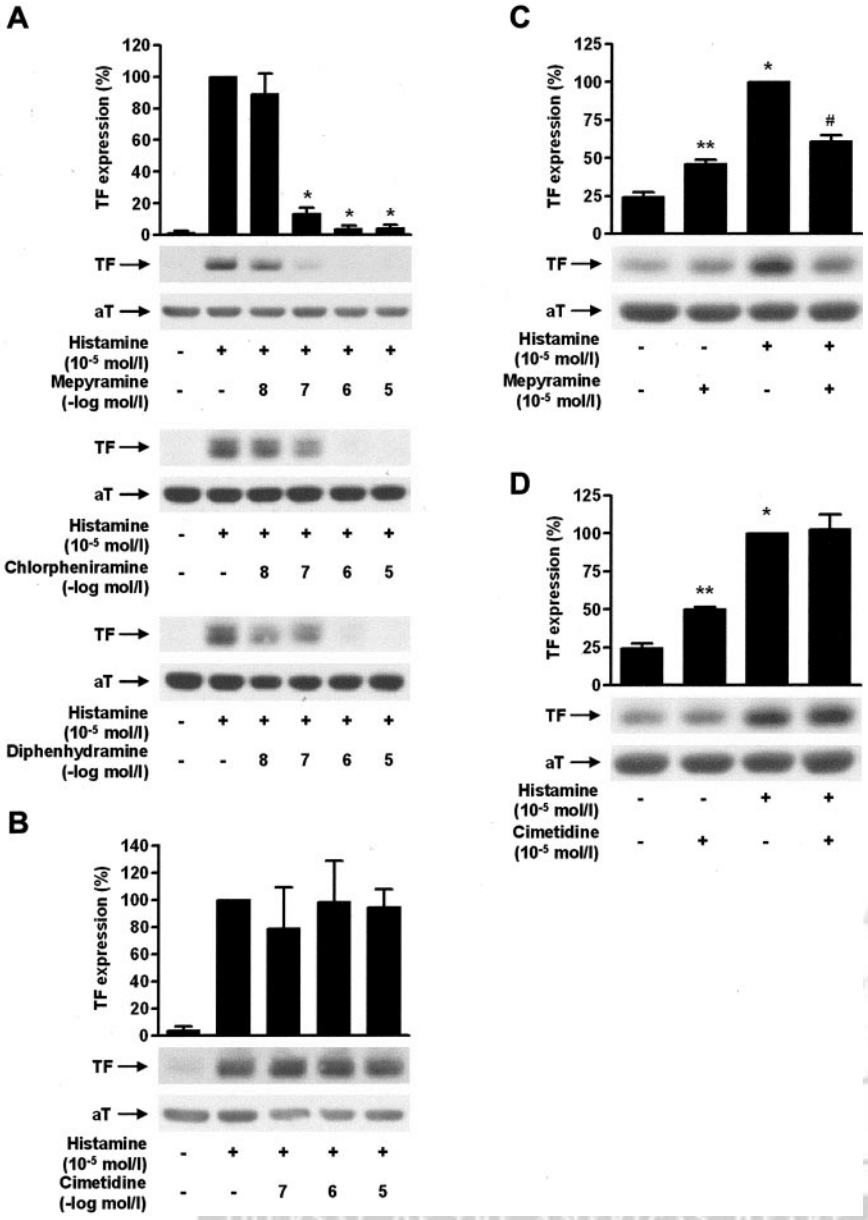


Figure 5. Histamine H_1 receptor, but not H_2 receptor, mediates induction of TF expression and surface activity. A, Mepyramine completely prevents histamine-induced TF expression in concentration-dependent manner in HAECs (top). *$P<0.0001$ vs histamine. A similar effect is observed with chlorpheniramine (middle) and diphenhydramine (bottom). B, Cimetidine does not affect histamine-induced TF expression in HAECs. C, Mepyramine impairs histamine-induced increase in TF expression in HAVSMCs. *$P<0.0001$ vs control; #$P<0.0001$ vs histamine; **$P<0.002$ vs control. D, Cimetidine does not affect histamine-induced TF expression in HAVSMCs. *$P<0.0001$ vs control; **$P<0.0002$ vs control. Blots are representative of 4 different experiments except for pretreatment with chlorpheniramine (n=2) and diphenhydramine (n=2). All blots are normalized to α-tubulin (aT) expression.

concentration-dependent manner to 38% of control (Figure 7B). Finally, SP600125 (10^{-7} to 10^{-6} mol/L), a specific inhibitor of JNK, diminished histamine-induced TF expression to 44% of control (Figure 7C). No cytotoxic effect of either of these drugs was observed at the concentrations used (n=3; P=NS; data not shown).

The Rho pathway is involved in the regulation of thrombin-induced TF expression. Therefore, the effect of Rho-kinase inhibitors on histamine-induced TF expression was examined. Pretreatment with Y-27632 (10^{-5} mol/L) or hydroxyfasudil (10^{-5} mol/L) did not result in a significant alteration of TF expression after stimulation with histamine (10^{-5} mol/L; Figure 8A). Activation of the phosphatidylinositol 3–kinase (PI3-kinase) pathway has been described in thrombin-induced TF expression.[22,24] Therefore, the effect of PI3-kinase inhibitors on histamine-induced TF expression was assessed. Pretreatment with LY294002 (5×10^{-6} mol/L) or wortmannin (10^{-7} mol/L) enhanced histamine-induced TF expression (Figure 8B).

Discussion

The present study demonstrates that histamine induces a concentration- and time-dependent increase in TF expression in vascular cells. Biologically active TF is located at the cell surface; indeed, stimulation of TF expression by histamine was paralleled by enhanced TF surface activity. The increase in the latter was less pronounced than that in TF protein expression; the distribution of TF in several cellular compartments or the expression of encrypted TF at the cell surface may account for this difference.[25,26] Unlike endothelial cells, unstimulated vascular smooth muscle cells displayed a relatively pronounced basal expression of TF, consistent with the rapid formation of a localized nidus of coagulation after endothelial denudation in vivo. This higher basal expression

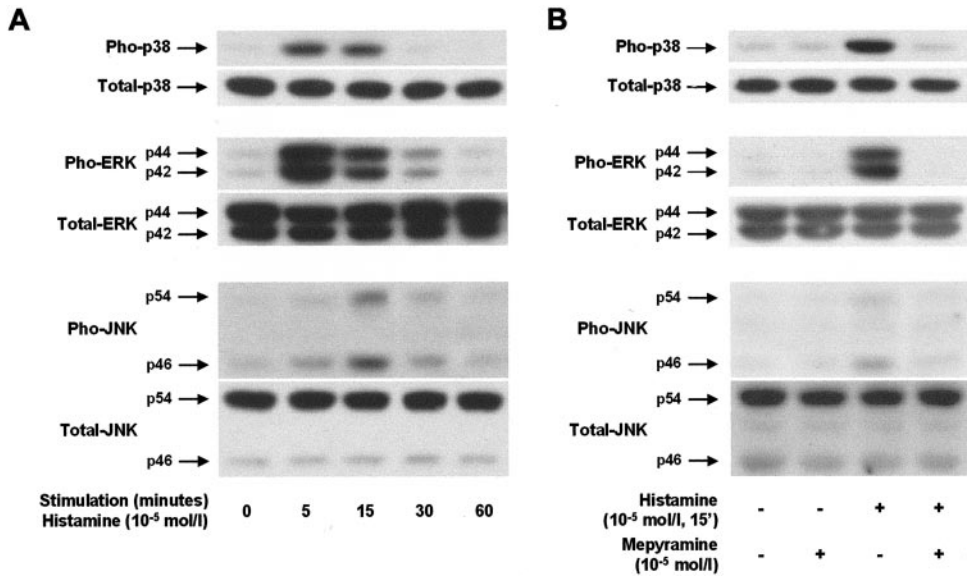


Figure 6. Histamine activates p38, ERK, and JNK via the H_1 receptor. A, Histamine induces transient, time-dependent activation of p38, ERK, and JNK. No change in expression of total levels of MAP kinases is observed. B, Blocking of H_1 receptor with mepyramine abolishes histamine-induced activation of p38, ERK, and JNK. Again, no change in expression of total levels of MAP kinases is observed. Blots are representative of 3 different experiments.

in HAVSMCs may account for the observation that a maximal increase in both TF expression and activity after stimulation with histamine was lower than in HAECs. The small but significant increase in TF expression in HAVSMCs after the addition of mepyramine or cimetidine compared with control conditions was probably due to partial agonistic activity of the respective antagonist.

TF expression is known to be regulated at the transcriptional level in response to many stimuli.[18,27] Consistent with these observations, histamine induced TF mRNA transcription in a time-dependent manner. Indeed, the kinetics of mRNA induction in response to histamine were similar to those observed with other mediators.[27,28] Moreover, the sequence of intracellular events after stimulation with histamine is consistent with a logical order: The maximum MAP kinase activation occurred after 5 to 15 minutes, RNA expression peaked after 1 hour, and protein expression reached its maximum after 5 hours.

Histamine-induced TF expression was compared with that in response to TNF-α, a known potent inducer of TF.[16,26] In both HAECs and HAVSMCs, histamine induced TF to more than one third of the level observed after stimulation with 10 ng/mL TNF-α. Because this concentration of TNF-α is very high, the effect of histamine compares well with that of the classic inducer of TF expression.

At the cellular level, TF expression is counterbalanced by TFPI. Several inducers of TF expression have been investigated with respect to their ability to affect TFPI expression; indeed, an increase, a decrease, or no alteration in TFPI expression has been observed after endothelial stimulation with TNF-α, LPS, and 5-hydroxytryptamine.[29–31] Stimulation of HAECs with histamine did not affect TFPI expression; thus, histamine-induced TF expression may result in particularly pronounced procoagulative changes because it is not paralleled by an increase in TFPI.

TF expression in HAECs and HAVSMCs is mediated by activation of the H_1 receptor. Indeed, activation of this receptor plays an important role in the pathogenesis of cardiovascular disease. Vascular contractions to histamine are mediated through activation of the H_1 receptor.[11,32] Histamine-induced P-selectin expression on endothelial cells, a key step in leukocyte transmigration to the subendothelium, also is mediated by this receptor.[33] Moreover, chlorpheniramine, a specific H_1 receptor blocker used in our study, prevents lesion formation in a rabbit model of atherosclerosis,[34] and the H_1 receptor blocker diphenhydramine, but not the H_2-receptor blocker cimetidine, prevents intimal thickening after vascular injury.[35] Consistent with these observations, expression of the H_1 receptor is increased in endothelial and vascular smooth muscle cells from atherosclerotic vessels.[36] Hence, the effect of histamine on TF expression adds to the evidence that histamine-induced vascular dysfunction is mediated through activation of the H_1 receptor.

Histamine leads to activation of p38, ERK, and JNK via the H_1 receptor. Consistent with our observation, activation of MAP kinases has been demonstrated in vascular cells in response to histamine.[37,38] Indeed, activation of p38 and ERK is mediated through the H_1 receptor in a smooth muscle cell line.[37] Other inflammatory agents such as TNF-α are known to activate all 3 MAP kinases in vascular cells.[16,23] With respect to TF expression, the pattern of MAP kinase activation in vascular cells is largely dependent on the specific stimulus. p38 is involved in thrombin-induced TF expression in endothelial cells.[22] p38 and ERK mediate TNF-α- and vascular endothelial growth factor–induced TF expression in endothelial cells.[23] Furthermore, JNK mediates TF expression in endothelial cells stimulated with TNF-α.[16] In contrast, ERK, but not p38, regulates lysophosphatic acid–induced TF expression in vascular smooth muscle cells.[19] Interestingly, in both LPS-stimulated monocytes and thrombin-stimulated en-

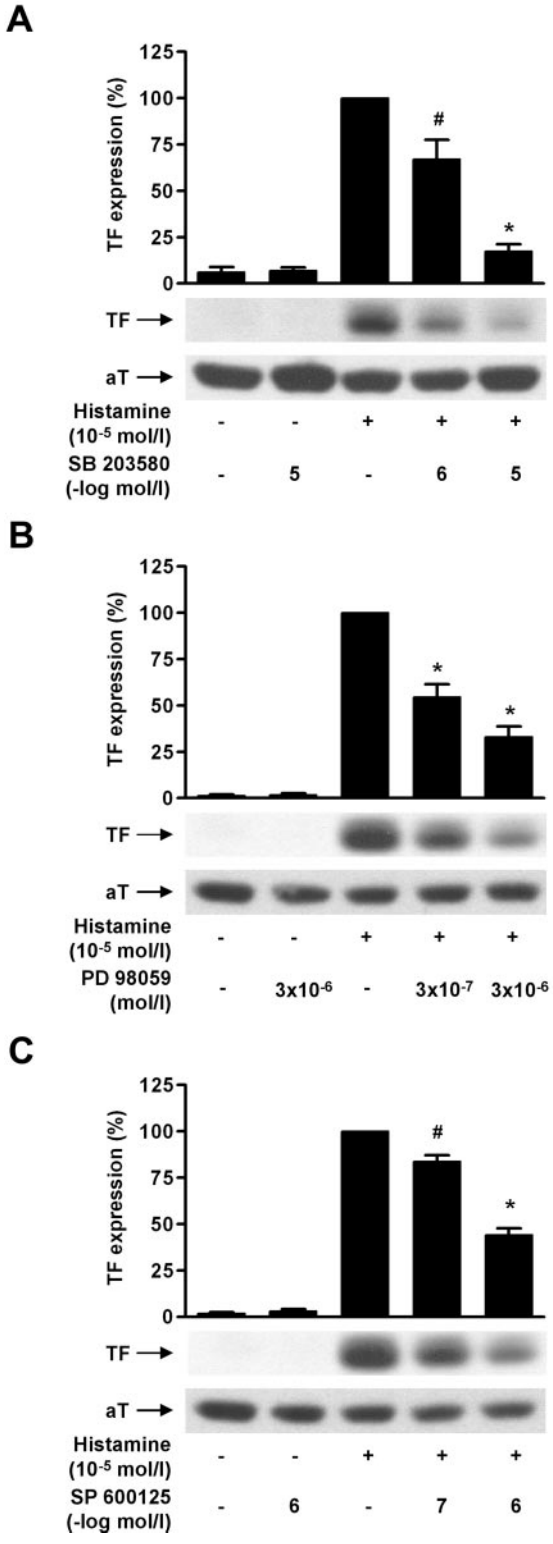


Figure 7. Inhibition of p38, ERK, and JNK impairs histamine-induced TF expression. A, SB203580, specific inhibitor of p38 activation, impairs histamine-induced TF expression. #$P<0.02$, *$P<0.0001$ vs histamine. B, PD98059, specific inhibitor of ERK activation, impairs histamine-induced TF expression. *$P<0.0001$ vs histamine. C, SP600125, specific inhibitor of JNK activation, impairs histamine-induced TF expression. #$P<0.01$, *$P<0.0001$ vs histamine. No change in basal TF expression is observed after application of SB203580, PD98059, or SP600125 compared with unstimulated control. Blots are representative of 4 different experiments; all blots are normalized to α-tubulin (aT) expression.

dothelial cells, TF expression occurs through activation of all 3 MAP kinase pathways.[27,39] We show here that specific inhibition of any of the 3 MAP kinases leads to a marked reduction in histamine-induced TF expression in HAECs even at low concentrations of the respective inhibitor. Thus, similar to LPS-stimulated monocytes,[27] activation of p38, ERK, and JNK cooperatively regulates histamine-induced TF expression in HAECs. Inhibition of p38 has beneficial effects on cardiac ischemia, reperfusion, endothelial function, and hypertensive end-organ damage.[40,41] Hence, our study implicates that inhibiting MAP kinases might represent a potential target for the treatment of thrombotic vascular disease. Nevertheless, the use of MAP kinase inhibitors in vivo is still controversial and requires further study.[41,42]

RhoA activation plays a major role in thrombin-induced TF expression in HAECs.[22,24] Indeed, histamine exerts some of its effects on endothelial cells through activation of the Rho pathway.[43,44] Surprisingly, Rho-kinase inhibition did not alter TF expression in response to histamine. Hence, the Rho pathway does not seem to be involved in histamine-induced TF expression. Indeed, RhoA activation may be affected by the cell type and/or the specific stimulus involved; alternatively, cell culture conditions may play a role, as has been implied in the regulation of cell permeability by histamine.[43,44] PI3-kinase inhibition enhances thrombin- and TNF-α–stimulated TF expression in endothelial cells and monocytes, respectively.[22,24,27] Consistent with these observations, histamine-induced TF expression was further increased after inhibition of PI3-kinase. Hence, similar to TF expression stimulated by other mediators, the PI-3 kinase pathway also plays a negative regulatory role in histamine-induced TF expression.

Histamine is released from mast cells, endothelial cells, and activated platelets and elicits potent arterial contractions.[1–3,11,32,45] Vasospasm mainly occurs in coronary arteries exhibiting some atherosclerotic changes and is promoted by the presence of mast cells and dysfunctional endothelial cells. Coronary artery vasospasm is involved in variant angina and has been implicated in coronary artery thrombus formation.[12–14] The latter is indeed crucial for the pathogenesis of arterial occlusion, and TF is a key enzyme for its initiation. As histamine induces TF expression, the release of the amine from mast cells or endothelial cells may represent an explantation for the frequent simultaneous occurrence of atherosclerosis, vasospasm, and thrombus formation. Moreover, the release of histamine from aggregating platelets may function as a positive feedback mechanism, further enhancing TF expression and thus thrombus formation. Interestingly, histamine levels are increased in platelets from patients with peripheral artery disease.[46]

Augmented TF expression in HAECs and HAVSMCs after stimulation with histamine is consistent with atherosclerosis as an inflammatory disease.[9,15] Indeed, elevated levels of TF have been observed in patients with hypertension, dyslipidemia, diabetic vasculopathy, and peripheral artery disease.[15,47–50] Not surprisingly, TF has also been implicated in coronary artery disease and acute myocardial infarction.[15] Here, we show that H_1 receptor antagonists completely abolish histamine-induced TF expression in HAECs. Hence,

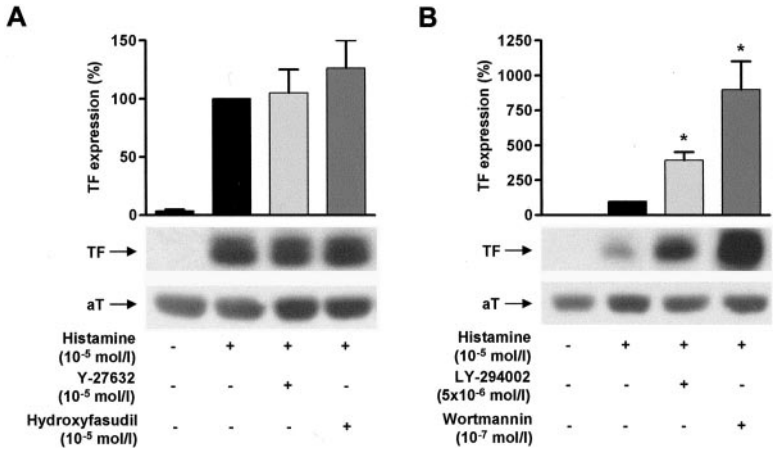


Figure 8. Role of Rho kinase and PI3-kinase pathway in histamine-induced TF expression. A, Preincubation with Rho-kinase inhibitor Y-27632 or hydroxyfasudil does not alter histamine-induced TF expression. B, PI3-kinase inhibitors LY294002 and wortmannin enhance histamine-induced TF expression 4- and 9-fold, respectively. *$P<0.01$ vs histamine. Values are given as fold increase in TF expression compared with histamine alone. Blots are representative of 3 different experiments; all blots are normalized to α-tubulin (aT) expression.

our findings suggest a therapeutic potential of H_1 receptor antagonists in the treatment of acute coronary syndromes.

In summary, histamine-induced TF expression may mediate thrombus formation in atherogenesis, especially in inflammation and vasospasm, implicating interesting novel perspectives for the treatment of vascular occlusive diseases such as acute coronary syndromes.

Acknowledgments

This work was supported by the Swiss National Science Foundation (grant 3200B0-102232/1 to Dr Tanner and grant 3100-068118.02/1 to Dr Lüscher), the Hartmann-Müller Foundation, the Herzog-Egli Foundation, and the Olga Mayenfisch Foundation. We thank Hana Joch for expert technical support.

References

1. Hollis TM, Gallik SG, Orlidge A, Yost JC. Aortic endothelial and smooth muscle histamine metabolism: relationship to aortic ^{125}I-albumin accumulation in experimental diabetes. *Arteriosclerosis*. 1983;3:599–606.
2. Brown MJ, Ind PW, Jenner DA. Platelet histamine. *N Engl J Med*. 1980;303:756.
3. Mannaioni PF, Di Bello MG, Masini E. Platelets and inflammation: role of platelet-derived growth factor, adhesion molecules and histamine. *Inflamm Res*. 1997;46:4–18.
4. Lee M, Sommerhoff CP, von Eckardstein A, Zettl F, Fritz H, Kovanen PT. Mast cell tryptase degrades HDL and blocks its function as an acceptor of cellular cholesterol. *Arterioscler Thromb Vasc Biol*. 2002;22:2086–2091.
5. Atkinson JB, Harlan CW, Harlan GC, Virmani R. The association of mast cells and atherosclerosis: a morphologic study of early atherosclerotic lesions in young people. *Hum Pathol*. 1994;25:154–159.
6. Kaartinen M, van der Wal AC, van der Loos CM, Piek JJ, Koch KT, Becker AE, Kovanen PT. Mast cell infiltration in acute coronary syndromes: implications for plaque rupture. *J Am Coll Cardiol*. 1998;32:606–612.
7. Laine P, Kaartinen M, Penttila A, Panula P, Paavonen T, Kovanen PT. Association between myocardial infarction and the mast cells in the adventitia of the infarct-related coronary artery. *Circulation*. 1999;99:361–369.
8. Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med*. 1999;340:115–126.
9. Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420:868–874.
10. Forman MB, Oates JA, Robertson D, Robertson RM, Roberts LJ 2nd, Virmani R. Increased adventitial mast cells in a patient with coronary spasm. *N Engl J Med*. 1985;313:1138–1141.
11. Shimokawa H, Tomoike H, Nabeyama S, Yamamoto H, Araki H, Nakamura M, Ishii Y, Tanaka K. Coronary artery spasm induced in atherosclerotic miniature swine. *Science*. 1983;221:560–562.
12. Oliva PB, Breckinridge JC. Arteriographic evidence of coronary arterial spasm in acute myocardial infarction. *Circulation*. 1977;56:366–374.
13. Buja LM, Hillis LD, Petty CS, Willerson JT. The role of coronary arterial spasm in ischemic heart disease. *Arch Pathol Lab Med*. 1981;105:221–226.
14. Hackett D, Davies G, Chierchia S, Maseri A. Intermittent coronary occlusion in acute myocardial infarction: value of combined thrombolytic and vasodilator therapy. *N Engl J Med*. 1987;317:1055–1059.
15. Moons AH, Levi M, Peters RJ. Tissue factor and coronary artery disease. *Cardiovasc Res*. 2002;53:313–325.
16. Steffel J, Hermann M, Greutert H, Gay S, Luscher TF, Ruschitzka F, Tanner FC. Celecoxib decreases endothelial tissue factor expression through inhibition of c-Jun terminal NH2 kinase phosphorylation. *Circulation*. 2005;111:1685–1689.
17. Tanner FC, Yang ZY, Duckers E, Gordon D, Nabel GJ, Nabel EG. Expression of cyclin-dependent kinase inhibitors in vascular disease. *Circ Res*. 1998;82:396–403.
18. Bogdanov VY, Balasubramanian V, Hathcock J, Vele O, Lieb M, Nemerson Y. Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. *Nat Med*. 2003;9:458–462.
19. Cui MZ, Zhao G, Winokur AL, Laag E, Bydash JR, Penn MS, Chisolm GM, Xu X. Lysophosphatidic acid induction of tissue factor expression in aortic smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2003;23:224–230.
20. Levi-Schaffer F, Temkin V, Malamud V, Feld S, Zilberman Y. Mast cells enhance eosinophil survival in vitro: role of TNF-alpha and granulocyte-macrophage colony-stimulating factor. *J Immunol*. 1998;160:5554–5562.
21. Gao B, Tsan MF. Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor alpha release by murine macrophages. *J Biol Chem*. 2003;278:174–179.
22. Eto M, Kozai T, Cosentino F, Joch H, Luscher TF. Statin prevents tissue factor expression in human endothelial cells: role of Rho/Rho-kinase and Akt pathways. *Circulation*. 2002;105:1756–1759.
23. Mechtcheriakova D, Schabbauer G, Lucerna M, Clauss M, De Martin R, Binder BR, Hofer E. Specificity, diversity, and convergence in VEGF and TNF-α signaling events leading to tissue factor up-regulation via EGR-1 in endothelial cells. *FASEB J*. 2001;15:230–242.
24. Viswambharan H, Ming XF, Zhu S, Hubsch A, Lerch P, Vergeres G, Rusconi S, Yang Z. Reconstituted high-density lipoprotein inhibits thrombin-induced endothelial tissue factor expression through inhibition of RhoA and stimulation of phosphatidylinositol 3-kinase but not Akt/endothelial nitric oxide synthase. *Circ Res*. 2004;94:918–925.
25. Rao LV, Pendurthi UR. Tissue factor on cells. *Blood Coagul Fibrinolysis*. 1998;9(suppl 1):S27–S35.
26. Schecter AD, Spirn B, Rossikhina M, Giesen PL, Bogdanov V, Fallon JT, Fisher EA, Schnapp LM, Nemerson Y, Taubman MB. Release of active tissue factor by human arterial smooth muscle cells. *Circ Res*. 2000;87:126–132.
27. Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J Biol Chem*. 2002;277:32124–32132.
28. Bartha K, Brisson C, Archipoff G, de la Salle C, Lanza F, Cazenave JP, Beretz A. Thrombin regulates tissue factor and thrombomodulin mRNA

levels and activities in human saphenous vein endothelial cells by distinct mechanisms. *J Biol Chem*. 1993;268:421–429.
29. Shimokawa T, Yamamoto K, Kojima T, Saito H. Down-regulation of murine tissue factor pathway inhibitor mRNA by endotoxin and tumor necrosis factor-alpha in vitro and in vivo. *Thromb Res*. 2000;100: 211–221.
30. Ameri A, Kuppuswamy MN, Basu S, Bajaj SP. Expression of tissue factor pathway inhibitor by cultured endothelial cells in response to inflammatory mediators. *Blood*. 1992;79:3219–3226.
31. Kawano H, Tsuji H, Nishimura H, Kimura S, Yano S, Ukimura N, Kunieda Y, Yoshizumi M, Sugano T, Nakagawa K, Masuda H, Sawada S, Nakagawa M. Serotonin induces the expression of tissue factor and plasminogen activator inhibitor-1 in cultured rat aortic endothelial cells. *Blood*. 2001;97:1697–1702.
32. Okumura K, Yasue H, Matsuyama K, Morikami Y, Ogawa H, Obata K. Effect of H1 receptor stimulation on coronary artery diameter in patients with variant angina: comparison with effect of acetylcholine. *J Am Coll Cardiol*. 1991;17:338–345.
33. Asako H, Kurose I, Wolf R, DeFrees S, Zheng ZL, Phillips ML, Paulson JC, Granger DN. Role of H1 receptors and P-selectin in histamine-induced leukocyte rolling and adhesion in postcapillary venules. *J Clin Invest*. 1994;93:1508–1515.
34. Harman D. Atherosclerosis: inhibiting effect of an antihistaminic drug, chlorpheniramine. *Circ Res*. 1962;11:277–282.
35. Miyazawa N, Watanabe S, Matsuda A, Kondo K, Hashimoto H, Umemura K, Nakashima M. Role of histamine H1 and H2 receptor antagonists in the prevention of intimal thickening. *Eur J Pharmacol*. 1998;362:53–59.
36. Takagishi T, Sasaguri Y, Nakano R, Arima N, Tanimoto A, Fukui H, Morimatsu M. Expression of the histamine H1 receptor gene in relation to atherosclerosis. *Am J Pathol*. 1995;146:981–988.
37. Robinson AJ, Dickenson JM. Activation of the p38 and p42/p44 mitogen-activated protein kinase families by the histamine H(1) receptor in DDT(1)MF-2 cells. *Br J Pharmacol*. 2001;133:1378–1386.
38. Gudmundsdottir IJ, Halldorsson H, Magnusdottir K, Thorgeirsson G. Involvement of MAP kinases in the control of cPLA(2) and arachidonic acid release in endothelial cells. *Atherosclerosis*. 2001;156:81–90.
39. Liu Y, Pelekanakis K, Woolkalis MJ. Thrombin and TNF alpha synergistically stimulate tissue factor expression in human endothelial cells: regulation through c-Fos and c-Jun. *J Biol Chem*. 2004;279:36142–36147.
40. Sanada S, Kitakaze M, Papst PJ, Hatanaka K, Asanuma H, Aki T, Shinozaki Y, Ogita H, Node K, Takashima S, Asakura M, Yamada J, Fukushima T, Ogai A, Kuzuya T, Mori H, Terada N, Yoshida K, Hori M. Role of phasic dynamism of p38 mitogen-activated protein kinase activation in ischemic preconditioning of the canine heart. *Circ Res*. 2001; 88:175–180.
41. Behr TM, Berova M, Doe CP, Ju H, Angermann CE, Boehm J, Willette RN. p38 mitogen-activated protein kinase inhibitors for the treatment of chronic cardiovascular disease. *Curr Opin Investig Drugs*. 2003;4: 1059–1064.
42. Ravingerova T, Barancik M, Strniskova M. Mitogen-activated protein kinases: a new therapeutic target in cardiac pathology. *Mol Cell Biochem*. 2003;247:127–138.
43. Wojciak-Stothard B, Ridley AJ. Rho GTPases and the regulation of endothelial permeability. *Vascul Pharmacol*. 2002;39:187–199.
44. van Nieuw Amerongen GP, Draijer R, Vermeer MA, van Hinsbergh VW. Transient and prolonged increase in endothelial permeability induced by histamine and thrombin: role of protein kinases, calcium, and RhoA. *Circ Res*. 1998;83:1115–1123.
45. Yang ZH, Diederich D, Schneider K, Siebenmann R, Stulz P, von Segesser L, Turina M, Buhler FR, Luscher TF. Endothelium-derived relaxing factor and protection against contractions induced by histamine and serotonin in the human internal mammary artery and in the saphenous vein. *Circulation*. 1989;80:1041–1048.
46. Gill DS, Barradas MA, Fonseca VA, Gracey L, Dandona P. Increased histamine content in leukocytes and platelets of patients with peripheral vascular disease. *Am J Clin Pathol*. 1988;89:622–626.
47. Felmeden DC, Spencer CG, Chung NA, Belgore FM, Blann AD, Beevers DG, Lip GY. Relation of thrombogenesis in systemic hypertension to angiogenesis and endothelial damage/dysfunction (a substudy of the Anglo-Scandinavian Cardiac Outcomes Trial [ASCOT]). *Am J Cardiol*. 2003;92:400–405.
48. Blann AD, Amiral J, McCollum CN, Lip GY. Differences in free and total tissue factor pathway inhibitor, and tissue factor in peripheral artery disease compared to healthy controls. *Atherosclerosis*. 2000; 152:29–34.
49. Lim HS, Blann AD, Lip GY. Soluble CD40 ligand, soluble P-selectin, interleukin-6, and tissue factor in diabetes mellitus: relationships to cardiovascular disease and risk factor intervention. *Circulation*. 2004;109: 2524–2528.
50. Sambola A, Osende J, Hathcock J, Degen M, Nemerson Y, Fuster V, Crandall J, Badimon JJ. Role of risk factors in the modulation of tissue factor activity and blood thrombogenicity. *Circulation*. 2003;107: 973–977.

American Heart
Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Journal of Thrombosis and Haemostasis, **4**: 2452–2460

ORIGINAL ARTICLE

Histamine differentially interacts with tumor necrosis factor-α and thrombin in endothelial tissue factor induction: the role of c-Jun NH_2-terminal kinase

J. STEFFEL,*†‡ C. ARNET,*† A. AKHMEDOV,*† S. M. ISELI,*† T. F. LÜSCHER*†‡ and F. C. TANNER*†‡
*Cardiovascular Research, Physiology Institute, University of Zürich; †Center for Integrative Human Physiology, University of Zürich; and ‡Cardiology, Cardiovascular Center, University Hospital Zürich, Zürich, Switzerland

To cite this article: Steffel J, Arnet C, Akhmedov A, Iseli SM, Lüscher TF, Tanner FC. Histamine differentially interacts with tumor necrosis factor-α and thrombin in endothelial tissue factor induction: the role of c-Jun NH_2-terminal kinase. *J Thromb Haemost* 2006; **4**: 2452–60.

Summary. *Background:* Histamine plays an important role in vascular disease. Tissue factor (TF) expression is induced in vascular inflammation and acute coronary syndromes. *Objectives:* This study examined the effect of histamine on tumor necrosis factor-α- (TNF-α-) vs. thrombin-induced endothelial TF expression. *Methods and results:* Histamine (10^{-8}–10^{-5} mol L^{-1}), TNF-α (5 ng mL^{-1}), and thrombin (1 U mL^{-1}) induced TF expression in human endothelial cells. Although TF expression by TNF-α and thrombin was identical, histamine augmented TNF-α-induced expression 7.0-fold, but thrombin-induced expression only 2.6-fold. Similar responses occurred with TF activity. The H_1-receptor antagonist mepyramine abrogated these effects. Differential augmentation by histamine was also observed at the mRNA level. Histamine-induced p38 activation preceded a weak second activation to both TNF-α and thrombin. Histamine-induced c-Jun NH_2-terminal kinase (JNK) activation was followed by a strong second activation to TNF-α, and less to thrombin. Selective inhibition of this second JNK activation by SP600125 reduced TF induction to histamine plus TNF-α by 67%, but to histamine plus thrombin by only 32%. Histamine augmented TNF-α- and thrombin-induced vascular cell adhesion molecule 1 (VCAM-1) expression to a similar extent. Consistent with this observation, VCAM-1 induction to TNF-α and thrombin was mediated by p38, but not by JNK. *Conclusions:* Histamine differentially augments TNF-α- vs. thrombin-induced TF expression and activity, which is mediated by the H_1-receptor, occurs at the mRNA level, and is related to differential JNK activation.

Correspondence: Felix C. Tanner, Cardiovascular Research, Physiology Institute, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland.
Tel.: +41 44 635 6469; fax: +41 44 635 6827; e-mail: felix.tanner@access.unizh.ch

Received 21 June 2006, accepted 21 August 2006

Keywords: inflammation, mitogen-activated protein kinase, signal transduction, thrombosis, tissue factor, vascular cell adhesion molecule-1.

Introduction

Histamine is involved in vascular inflammation and is released by mast cells, endothelial cells, and aggregating platelets [1–3]. Mast cell activation occurs in atherogenesis and coronary artery disease [4–7]; these cells are detected at high numbers in atherosclerotic plaques and also in coronary atherectomy specimens from patients with acute myocardial infarction [8,9]. Consistent with a role of histamine in vascular disease, mice deficient in histidine decarboxylase, the rate-limiting enzyme for histamine formation, exhibit reduced intimal thickening with a lower intima-to-media ratio after vascular injury [10]. Moreover, histidine decarboxylase and the histamine H_1-receptor are both expressed in atherosclerotic human coronary arteries, and granulocyte–macrophage colony-stimulating factor induces their expression in monocytes [11]. Activated mast cells also secrete other mediators known to play a role in inflammation and atherogenesis, such as tumor necrosis factor-α (TNF-α), so that concomitant release of histamine and TNF-α may well occur under these conditions [5].

Tissue factor (TF) is a key protein in the activation of coagulation and plays an important role in the pathogenesis of thrombotic diseases like acute coronary syndromes [12]. Indeed, TF antigen and activity are elevated in plasma and atherectomy specimens of patients with unstable angina. Moreover, TF may be involved in the pathogenesis of atherosclerosis, and different cell types in atherosclerotic plaques including endothelial cells exhibit a pronounced expression of TF [12]. In the inflammatory environment of atherosclerotic vessels, endothelial TF expression and activity is induced by mediators such as histamine and TNF-α [13]. As both mediators are released by mast cells, interactions of histamine with TNF-α regarding TF induction are likely to occur. Thrombin not only catalyzes the conversion of fibrinogen to fibrin, but also induces TF as a positive feedback

© 2006 International Society on Thrombosis and Haemostasis

mechanism. Therefore, interactions of histamine with thrombin may be important for regulation of TF expression as well.

This study was designed to investigate interactions of histamine with TNF-α vs. thrombin in endothelial TF expression.

Materials and methods

Cell culture

Human aortic endothelial cells (HAEC) were cultured as described [14]. Cells were grown to confluence in 6-cm culture dishes and rendered quiescent for 24 h before stimulation with histamine, TNF-α, or thrombin (all from Sigma, Munich, Germany). Histamine was preincubated for 1 h before stimulation with TNF-α or thrombin. Mepyramine and cimetidine (both from Sigma) were added to the dishes 30 min prior to stimulation. SB203580 was obtained from Sigma, SP600125 from Calbiochem (San Diego, CA, USA). To assess cytotoxicity, a colorimetric assay for detection of lactate dehydrogenase was used according to the manufacturer's recommendations (Roche, Mannheim, Germany).

Western blot analysis

Protein expression was determined by Western blot analysis as described [14,15]. Forty micrograms samples was separated by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE), and proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA) by semidry transfer. Antibodies against human TF (American Diagnostica, Pfungstadt, Germany) and human vascular cell adhesion molecule-1 (VCAM-1; R&D, Abingdon, UK) were used at 1:2000 dilution. Antibodies against phosphorylated p38 mitogen-activated protein kinase (MAPK) (p38) and phosphorylated c-Jun NH_2-terminal kinase (JNK; both from Cell Signaling, Allschwil, Switzerland) were used at 1:1000 dilution. Antibodies against total p38 (Cell Signaling) and total JNK (Cell Signaling and Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used at 1:2000 dilution. Blots were normalized to alpha-tubulin (aT) expression (1:10 000 dilution, Sigma).

Real time reverse transcriptase-polymerase chain reaction

Extraction of RNA from HAEC and conversion to cDNA was performed as described [13]. Real-time PCR was carried out in an MX3000P PCR cycler (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. All PCR experiments were performed using the SYBR Green JumpStart kit (Sigma). Each reaction (25 μL) contained 2 μL of cDNA, 1 pmol of each primer, 0.25 μL of internal reference dye, and 12.5 μL of JumpStart Taq ReadyMix (buffer, dNTPs, stabilizers, SYBR Green, Taq polymerase, and JumpStart Taq antibody). Primers for TF mRNA were used as described [13,16]. Primers for human L28: sense primer: 5′-GCATCTGCAATGGATGGT-3′; antisense primer: 5′-TGTTCTTGCGGATCATGTGT-3′. The amplification program consisted of 1 cycle at 95 °C for 10 min, followed by 40 cycles with a denaturing phase at 95 °C for 30 s, an annealing phase at 60 °C for 1 min, and an elongation phase at 72 °C for 1 min. A melting curve analysis was performed after amplification to verify the accuracy of the amplicon. PCR products were analyzed on an ethidium bromide stained 1% agarose gel for verification of the correct amplification. In each real-time PCR run for TF and L28, a calibration curve was included, which was generated from serial dilutions of a known TF and L28 standard, respectively.

Tissue factor surface activity

TF surface activity was analyzed using a colorimetric assay (American Diagnostica) according to the manufacturer's recommendations with some modifications as described [13,17]. Cells were grown in 12-well plates; after stimulation, cells were washed twice with phosphate-buffered saline followed by incubation with human factor (F) VIIa and FX at 37 °, allowing for the formation of TF/FVIIa-complex at the cell surface. TF/FVIIa-complex converted human FX to FXa, which was measured by its ability to metabolize a chromogenic substrate. Lipidated human TF was used as a positive control to confirm that the results obtained were in the linear range of detection (data not shown).

Statistics

Data are presented as mean + /-SEM. Unpaired Student's *t*-test was applied for comparison of two groups. A P-value < 0.05 was considered significant.

Results

Histamine differentially augments TNF-α- vs. thrombin-induced TF protein expression and activity

Stimulation of HAEC with histamine (10^{-8}–10^{-5} mol L^{-1}) induced TF protein expression in a concentration-dependent manner up to eighteenfold above the basal level (Fig. 1A) [13]. When cells were pretreated with histamine for 1 h, TNF-α-induced TF expression was augmented in a concentration-dependent manner; the maximal effect was observed with 10^{-5} mol L^{-1} histamine and accounted for a 7.0-fold increase in TF expression compared with TNF-α alone (Fig. 1B, left panel). Histamine also augmented thrombin-induced TF expression; in contrast to TNF-α, however, 10^{-5} mol L^{-1} histamine increased TF expression only 2.6-fold compared with thrombin alone (Fig. 1B, right panel). Consistent with this observation, histamine concentrations as low as 10^{-8} and 10^{-7} mol L^{-1}, which did not affect TF expression on their own, augmented TF expression in response to TNF-α, but not to thrombin (Fig. 1B). Direct comparison of the maximal histamine effect revealed a 3.5-fold higher augmentation of TNF-α as compared with thrombin-induced TF expression (Fig. 1C).

© 2006 International Society on Thrombosis and Haemostasis

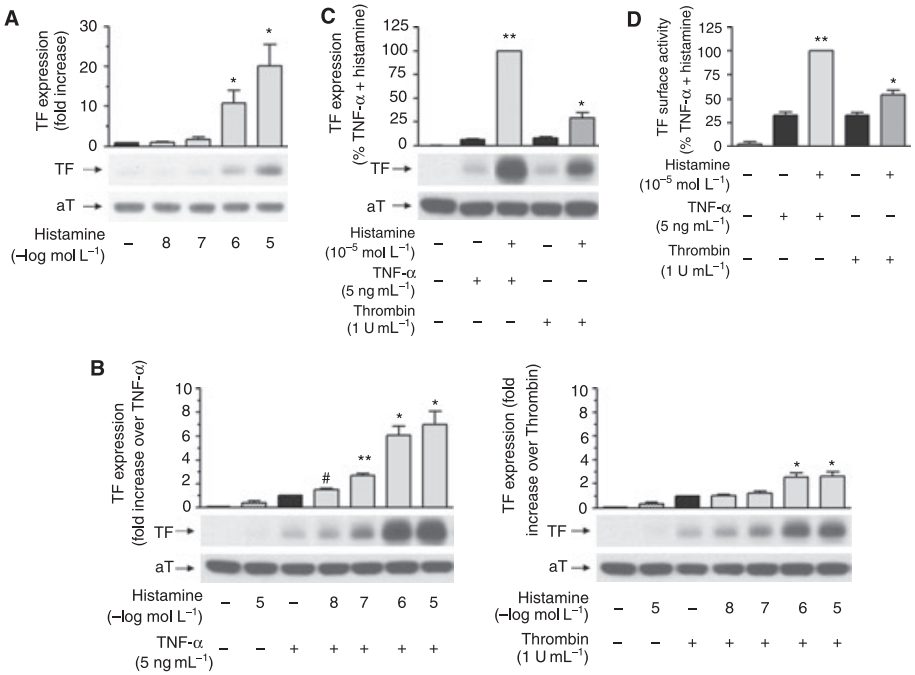


Fig. 1. Histamine differentially augments tumor necrosis factor-α- (TNF-α-) vs. thrombin-induced tissue factor (TF) protein expression and activity. (A) Histamine induces TF expression. Values are given as fold-increase over control. $*P < 0.01$ vs. control. (B) Left panel: histamine augments TNF-α-induced TF expression. $*P < 0.002$, $^{\#}P < 0.005$, $**P < 0.0001$ vs. TNF-α alone. Right panel: histamine augments thrombin-induced TF expression. $*P < 0.005$ vs. thrombin alone. (C) Histamine differentially augments TNF-α- vs. thrombin-induced TF expression. $**P < 0.0001$ vs. TNF-α alone and $P < 0.0001$ vs. thrombin plus histamine; $*P < 0.02$ vs. thrombin alone. (D) Histamine differentially augments TNF-α- and thrombin-induced TF surface activity. $**P < 0.0001$ vs. TNF-α alone and $P < 0.001$ vs. thrombin plus histamine; $*P < 0.02$ vs. thrombin alone. Cells were pretreated with histamine for 1 h before stimulation with TNF-α or thrombin for 5 h. Values are representative of four independent experiments except for TF surface activity ($n = 3$). Blots are normalized to alpha tubulin expression.

Importantly, the levels of TF induction by TNF-α alone vs. thrombin alone were identical (P = NS; Fig. 1B,C).

Similar to the pattern of TF protein expression, histamine differentially affected TF surface activity; the amine augmented TNF-α-induced TF activity 3.0-fold, but thrombin-induced activity only 1.7-fold (Fig. 1D). TF surface activity induced by TNF-α alone vs. thrombin alone was identical.

Histamine augments TNF-α- and thrombin-induced TF expression via the H_1-receptor

Histamine induces TF expression via the H_1-receptor [13]. The effect of histamine on both TNF-α- (Fig. 2A) and thrombin-induced TF expression (Fig. 2B) was abolished by mepyramine (10^{-5} mol L^{-1}), a selective H_1-receptor blocker, and no difference in TF expression was observed under these conditions compared with stimulation with TNF-α or thrombin alone (P = NS).

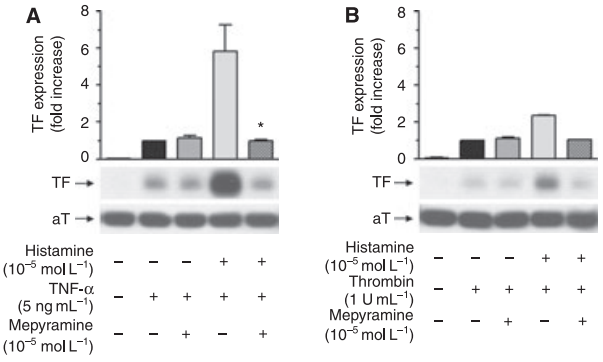


Fig. 2. Histamine augments tumor necrosis factor-α- (TNF-α-) and thrombin-induced tissue factor expression via the H_1-receptor. Mepyramine prevents the effect of histamine on (A)TNF-α-induced and (B) thrombin-induced TF expression. $*P < 0.05$ vs. TNF-α plus histamine and $*P$ = NS vs. TNF-α alone. Cells were pretreated with mepyramine for 1 h, followed by histamine for 1 h, and then stimulated with TNF-α or thrombin for 5 h. Values are representative of three (TNF-α) and two (thrombin) independent experiments. Blots are normalized to alpha tubulin expression.

Histamine differentially augments TNF-α- vs. thrombin-induced TF mRNA expression

The maximal increase of both TNF-α- and thrombin-induced TF protein expression was observed between 3 and 5 h (Fig. 3A). The augmenting effects of histamine on TNF-α- and thrombin-induced TF protein expression were maximal at the same time points (Fig. 3A). However, the effect of histamine on TNF-α-induced TF expression was severalfold higher than that on thrombin-induced expression at all time points examined (Fig. 3A).

© 2006 International Society on Thrombosis and Haemostasis

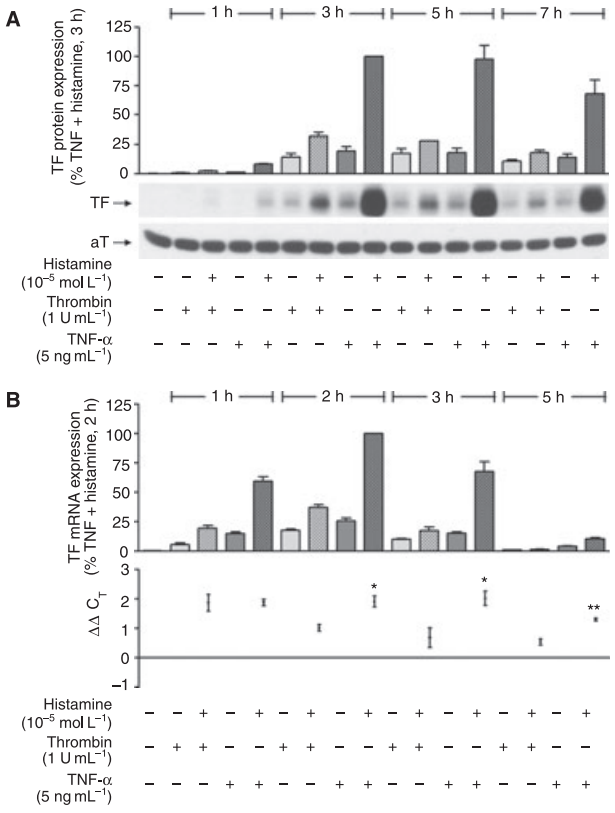


Fig. 3. Histamine differentially augments tumor necrosis factor-α- (TNF-α-) vs. thrombin-induced tissue factor (TF) mRNA expression. (A) Time course of TF protein expression. Differential augmentation of TNF-α- vs. thrombin-induced TF expression is observed at 3, 5, and 7 h. Values are representative of three independent experiments and normalized to alpha tubulin expression. (B) Time course of TF mRNA expression (real-time polymerase chain reaction). Differential augmentation of TNF-α- vs. thrombin-induced TF expression is observed at 2, 3, and 5 h. $*P < 0.02$; $**P < 0.001$. Cells were pretreated with histamine for 1 h before stimulation with TNF-α or thrombin for 5 h. Values are representative of four different experiments and normalized to L28 expression.

Real-time PCR analysis revealed that histamine augmented TNF-α-induced TF mRNA levels by 3.9-, 4.4-, and 2.6-fold after 2, 3, and 5 h of stimulation, respectively; in contrast, the amine augmented thrombin-induced TF levels by only 2.1-, 1.7-, and 1.6-fold after 2, 3, and 5 h of stimulation, respectively (Fig. 3B). Maximal expression of TF mRNA occurred after 2 h of stimulation under all conditions examined. Direct comparison of histamine's maximal effect revealed a 1.9-fold higher augmentation of TNF-α as compared with thrombin-induced TF mRNA levels (Fig. 3B).

TNF-α and thrombin differentially activate p38 and JNK after pretreatment with histamine

To analyze the role of p38 and JNK activation, HAEC were pretreated with either histamine (Fig. 4A, B, right panel) or carrier (H_2O; Fig. 4A, B, left panel) for 1 h before stimulation with TNF-α or thrombin. Histamine elicited a transient activation of p38 and JNK within the first hour of treatment (Fig. 4A, B) [13]. TNF-α alone and thrombin alone activated p38 and JNK as well (Fig. 4A, B, left panel). The kinetics of TNF-α- vs. thrombin-induced p38 phosphorylation were similar, with maximal activation occurring after 15 min with TNF-α and after 5 min with thrombin. Following preincubation with histamine, TNF-α and thrombin elicited a second activation of p38, which was weaker than that induced by histamine. In contrast to p38, the kinetics of JNK phosphorylation differed between TNF-α and thrombin: TNF-α activated JNK peaking after 15 min, while thrombin caused a gradual increase in JNK activation over 60 min (Fig. 4B, left panel). The effect of histamine preincubation on JNK activation also differed between TNF-α and thrombin. After pretreatment with histamine, JNK activation by TNF-α was quite pronounced, in comparison with both histamine alone and histamine plus thrombin; in contrast, JNK activation by thrombin was markedly weaker under these conditions (Fig. 4B, right panel).

JNK differentially mediates TNF-α- vs. thrombin-induced TF expression after pretreatment with histamine

MAPKs mediate TF induction in response to stimulation with histamine, TNF-α, and thrombin [13,14,18–20]. Inhibition of p38 by SB203580 and JNK by SP600125 decreased TNF-α-induced TF expression by 72% and 49%, respectively (Fig. 5A), while thrombin-induced TF expression was inhibited by 86% and 38%, respectively (Fig. 5B). Similarly, inhibition of p38 and JNK reduced histamine-augmented TF expression in response to TNF-α by 77% and 57%, respectively (Fig. 5C). In contrast, histamine-augmented TF expression in response to thrombin was reduced by 89% after inhibition of p38, but only by 26% after inhibition of JNK (Fig. 5D).

While TNF-α potently activated JNK after pretreatment with histamine, thrombin hardly affected JNK phosphorylation under the same conditions. Moreover, inhibition of JNK reduced TF expression in response to TNF-α plus histamine to a higher extent than that to thrombin plus histamine. To evaluate the significance of the second activation of JNK by TNF-α vs. thrombin after pretreatment with histamine, the JNK inhibitor SP600125 was added 45 min after stimulation with histamine. Under these conditions, no reduction in TF expression to histamine alone was observed (Fig. 6A). When cells were stimulated with TNF-α or thrombin 60 min after exposure to histamine (i.e. 15 min after treatment with SP600125), histamine-augmented TF expression was reduced by 67% in response to TNF-α (Fig. 6B), but only by 32% in response to thrombin (Fig. 6C).

Histamine does not differentially augment TNF-α vs. thrombin-induced VCAM-1 expression

Pretreatment with histamine augmented VCAM-1 expression in response to TNF-α by 69% (Fig. 7A, left panel) and to thrombin by 67% (Fig. 7A, right panel). Inhibition of p38 with SB203580 reduced TNF-α-induced VCAM-1 expression by

© 2006 International Society on Thrombosis and Haemostasis

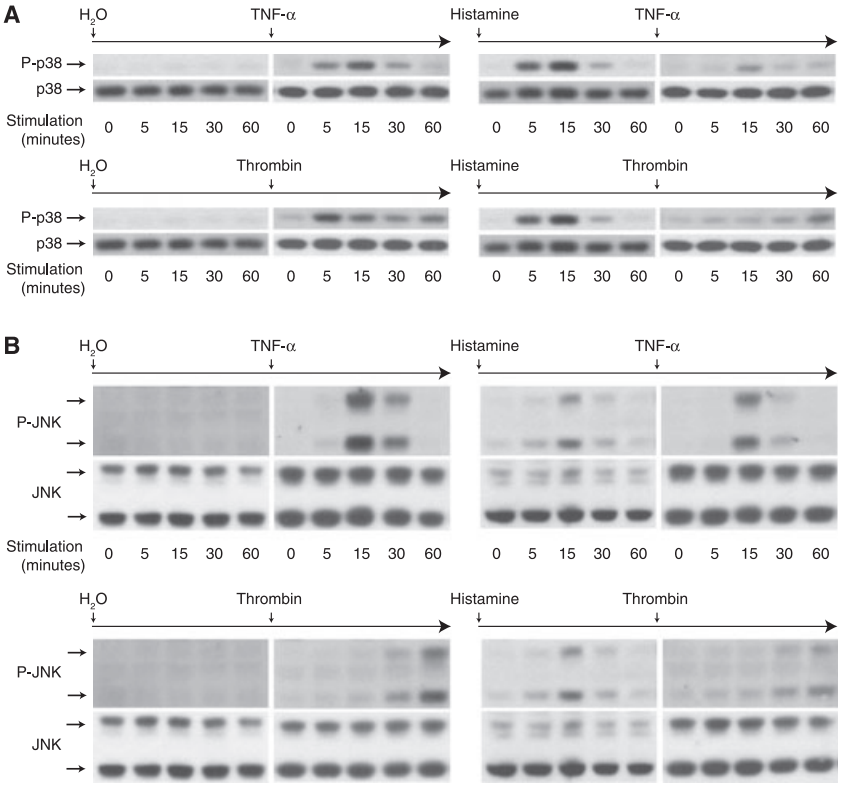


Fig. 4. Tumor necrosis factor-α (TNF-α) and thrombin differentially activate mitogen-activated protein kinases after pretreatment with histamine. HAECs are treated with carrier (H_2O, left panel) or histamine (right panel) for 1 h and then stimulated with TNF-α or thrombin for the time points indicated. Following treatment with histamine, the second wave of p38 activation by TNF-α or thrombin is weaker (A, right panel) than that without histamine (A, left panel). The second wave of c-Jun NH_2-terminal kinase (JNK) activation by TNF-α is quite pronounced, while that by thrombin is markedly weaker (B). Time courses of stimulation with TNF-α and TNF-α plus histamine, as well as with thrombin and thrombin plus histamine, are each performed and blotted in parallel, and blots are only separated for didactic reasons. There is no change in total protein expression of p38 and JNK. Blots are representative of three independent experiments (except for time course of H_2O stimulation [$n = 1$] and time course of histamine stimulation [$n = 1$], which was described previously) [13].

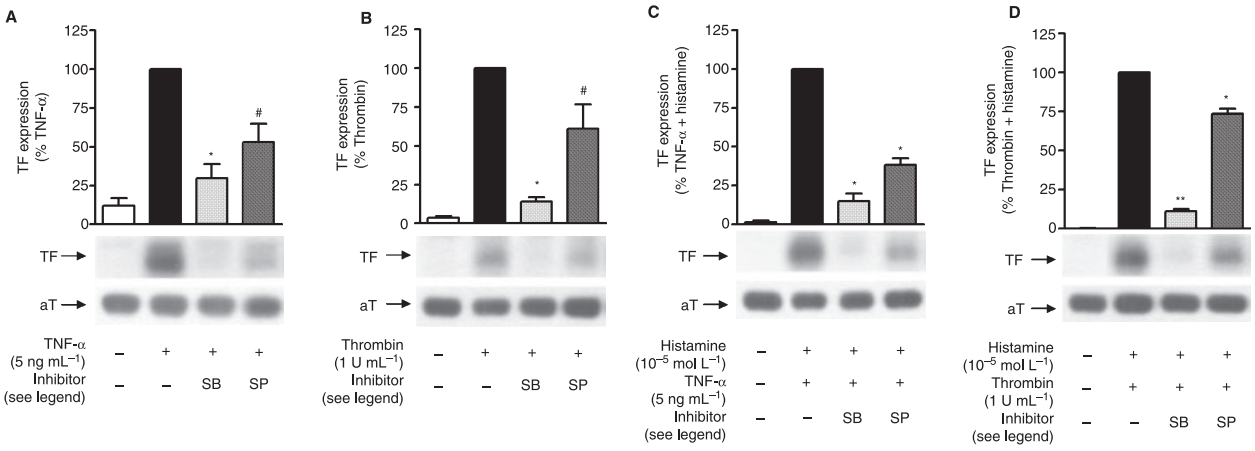


Fig. 5. Inhibition of p38 and c-Jun NH_2-terminal kinase (JNK) before pretreatment with histamine. (A) Tumor necrosis factor-α (TNF-α)-induced tissue factor (TF) expression is decreased by specific inhibition of p38 (SB203580) or JNK (SP600125) phosphorylation. $^{*}P < 0.0001$; $^{\#}P < 0.01$ vs. TNF-α alone. (B) Thrombin-induced TF expression is decreased by inhibition of p38 or JNK phosphorylation. $^{*}P < 0.0001$, $^{\#}P < 0.05$ vs. thrombin alone. Cells were pretreated with the inhibitors for 1 h and then stimulated with TNF-α or thrombin for 5 h. (C) Histamine-augmented TF expression to TNF-α is decreased by inhibition of p38 or JNK. $^{**}P < 0.0005$ vs. histamine plus TNF-α. (D) Histamine-augmented TF expression to thrombin is decreased by inhibition of p38 or JNK. Notably, the effect of SP600125 is much less pronounced than in (C). $^{**}P < 0.0001$, $^{*}P < 0.002$ vs. histamine plus thrombin. Cells were pretreated with the inhibitors for 1 h, followed by histamine for 1 h and then stimulated with TNF-α or thrombin for 5 h. Blots are representative of at least three different experiments and normalized to alpha tubulin expression. SB, SB203580 (10^{-5} mol L^{-1}); SP, SP600125 (10^{-6} mol L^{-1}).

© 2006 International Society on Thrombosis and Haemostasis

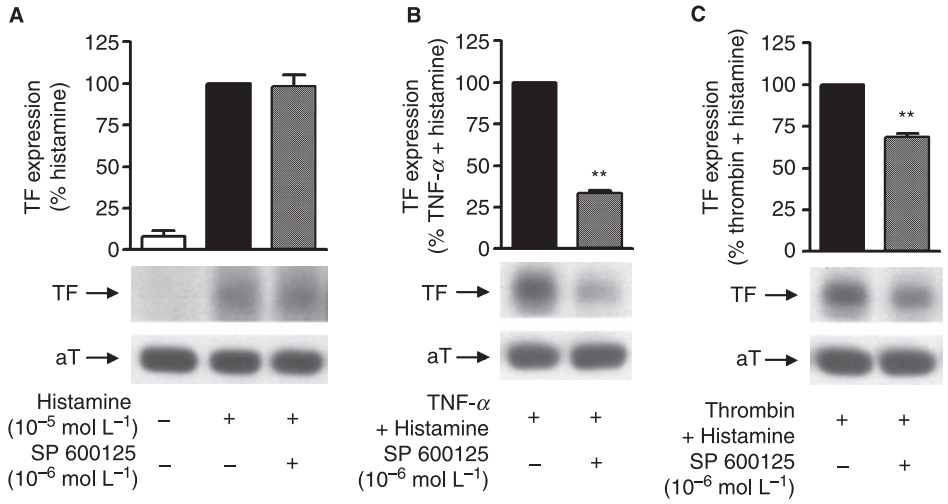


Fig. 6. Inhibition of c-Jun NH_2-terminal kinase (JNK) after pretreatment with histamine (A) Incubation of SP600125 (10^{-6} mol L^{-1}) 45 min after stimulation with histamine has no effect on histamine-induced tissue factor (TF) expression. (B) Incubation of SP600125 (10^{-6} mol L^{-1}) 45 min after pretreatment with histamine [i.e. 15 min before stimulation with tumor necrosis factor-α (TNF-α) for 5 h] reduces TNF-α-induced TF expression by 67% ($P < 0.0005$). (C) Incubation of SP600125 (10^{-6} mol L^{-1}) 45 min after pretreatment with histamine (i.e. 15 min before stimulation with thrombin for 5 h) reduces thrombin-induced TF expression by 32% ($P < 0.0005$).

60%, while inhibition of JNK with SP600125 had no significant effect (Fig. 7B). Inhibition of p38 reduced thrombin-induced VCAM-1 expression by 83%, while inhibition of JNK increased VCAM-1 expression (Fig. 7C). VCAM-1 expression in response to histamine plus TNF-α was reduced by 25% after inhibition of p38, while inhibition of JNK had no effect (Fig. 7D). VCAM-1 expression in response to histamine plus thrombin was reduced by 59% after inhibition of p38, while inhibition of JNK had no effect (Fig. 7E).

Discussion

This study demonstrates that histamine augments TNF-α-induced endothelial TF expression and activity in a much more potent manner than that stimulated by thrombin. These effects are restricted to TF, as histamine enhances TNF-α- vs. thrombin-induced VCAM-1 expression to the same extent.

Histamine induces endothelial TF expression via the H_1-receptor [13]. In line with this observation, the H_1-receptor antagonist mepyramine reduced the augmenting effect of histamine on both TNF-α- and thrombin-induced TF expression to the level observed with TNF-α or thrombin alone. Thus, the action of histamine on TNF-α and thrombin-induced TF expression is mediated via the H_1-receptor. Activation of this receptor plays an important role in the pathogenesis of vasospasm, vascular inflammation, and intimal thickening after vascular injury [21–23]. Thus, our data add to the growing evidence that histamine-induced vascular dysfunction is mediated through activation of the H_1-receptor.

Histamine, TNF-α, and thrombin are all known to upregulate TF expression at the transcriptional level [13,24,25]. Although histamine augmented TNF-α- and thrombin-induced TF mRNA expression, the effect on TNF-α-induced mRNA level was almost 2-fold higher than that on thrombin-induced mRNA expression. Thus, the differential action of histamine on TNF-α as compared with thrombin-induced TF expression occurred at the transcriptional level. Post-transcriptional actions contributing to this effect cannot be excluded completely; however, such effects are unlikely because the pattern of mRNA vs. protein expression was very similar.

Both p38 and JNK are involved in TF induction by histamine [13], TNF-α [14,18], and thrombin [18,26]. As the two MAPKs mediated TF induction by TNF-α and thrombin in both the presence and the absence of histamine, we sought to determine whether p38 or JNK could be involved in the differential augmenting effect of histamine on TNF-α- vs. thrombin-induced TF expression. Once histamine had activated p38, TNF-α and thrombin elicited a second activation, which was weaker than that by TNF-α or thrombin alone; thus, neither mediator could activate p38 a second time in a potent manner. Consistent with this observation, inhibition of p38 reduced TF induction by histamine plus TNF-α to a similar degree as that by histamine plus thrombin. These data indicate that p38 is not responsible for the differential augmenting effect of histamine on TNF-α as compared with thrombin-induced TF expression.

The pattern of JNK activation was not identical to that of p38. Indeed, once histamine had activated JNK, TNF-α elicited a second activation which was quite pronounced, while the second activation of JNK by thrombin was markedly weaker. This observation suggests that differential activation of JNK may predominantly be involved in the differential augmenting effect of histamine on TNF-α-induced as compared with thrombin-induced TF expression. In order to selectively eliminate the second wave of JNK activation, a specific JNK inhibitor was applied 45 min after histamine pretreatment (i.e. 15 min before stimulation with TNF-α or thrombin). Under these conditions, JNK inhibition did not

© 2006 International Society on Thrombosis and Haemostasis

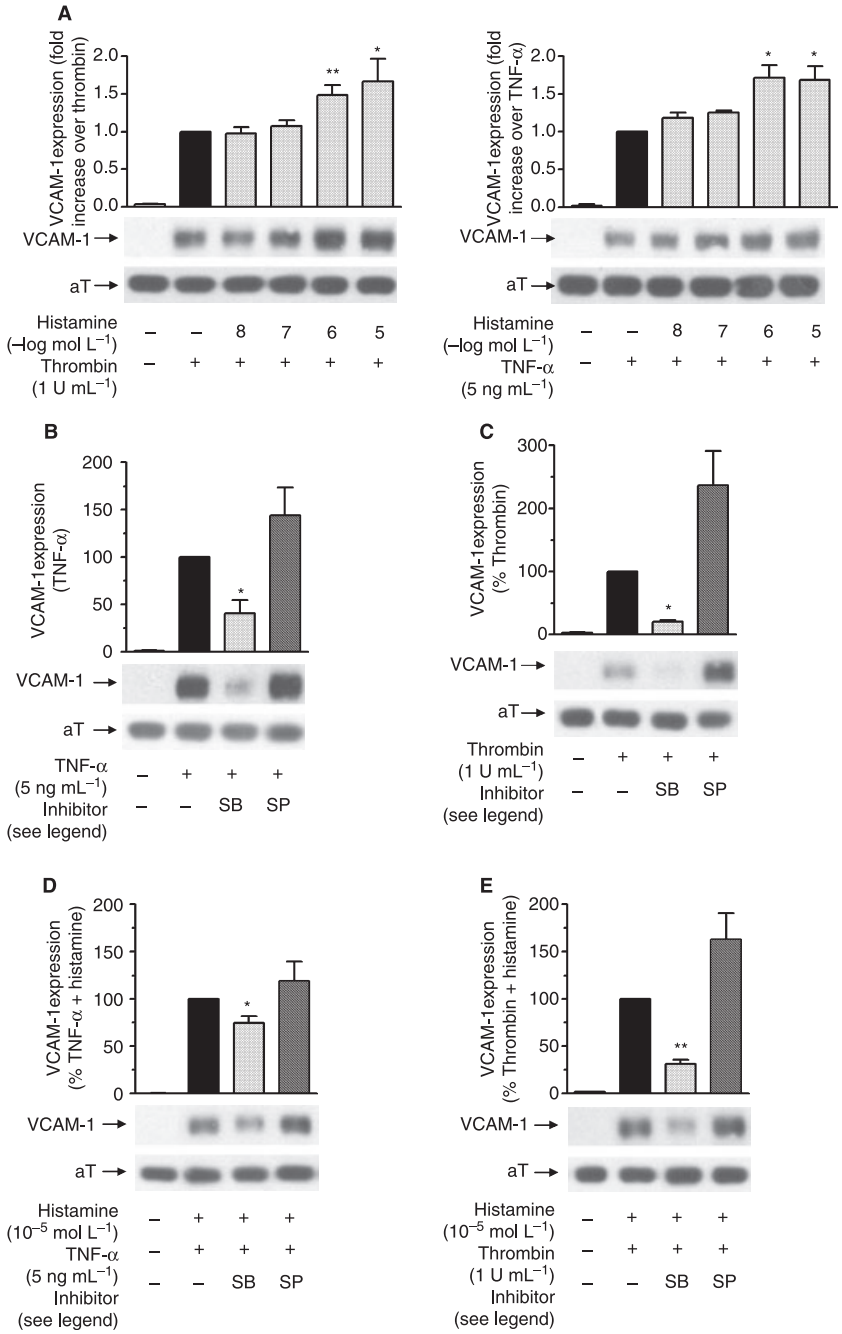


Fig. 7. Histamine does not differentially augment tumor necrosis factor-α- (TNF-α-) and thrombin-induced vascular cell adhesion molecule-1 (VCAM-1) expression. (A) Left panel: histamine enhances TNF-α-induced VCAM-1 expression. $*P < 0.05$ vs. TNF-α alone. Right panel: histamine enhances thrombin-induced VCAM-1 expression. $*P < 0.05$ vs. thrombin alone. (B) TNF-α-induced VCAM-1 expression is reduced by specific inhibition of p38, but not c-Jun NH_2-terminal kinase (JNK). $*P < 0.05$ vs. TNF-α. (C) Thrombin-induced VCAM-1 expression is reduced by inhibition of p38, but not JNK. $*P < 0.0001$, $^{\#}P < 0.05$ vs. thrombin. Cells were pretreated with the inhibitors for 1 h and then stimulated with TNF-α or thrombin for 5 h. (D) VCAM-1 expression in response to histamine plus TNF-α is reduced by inhibition of p38, but not JNK. $*P < 0.01$ vs. TNF-α plus histamine. (E) VCAM-1 expression to histamine plus thrombin is reduced by inhibition of p38, but not JNK. $*P < 0.0001$ vs. thrombin plus histamine. Cells were pretreated with the inhibitors for 1 h, followed by histamine for 1 h and then stimulated with TNF-α or thrombin for 5 h. Blots are representative of at least three different experiments and normalized to alpha-tubulin expression. SB, SB203580 (10^{-5} mol L^{-1}); SP, SP600125 (10^{-6} mol L^{-1}).

affect TF induction by histamine alone, indicating that the wave of activation occurring during the first 45 min after histamine stimulation fully accounts for the action of JNK on histamine-induced TF expression. When cells were then stimulated with TNF-α in the presence of the JNK inhibitor, the augmenting effect of histamine on TF expression was markedly reduced; however, when they were stimulated with thrombin, the reduction was much less pronounced. This strongly suggests that JNK plays a major role in the differential augmentation of TF expression by histamine.

© 2006 International Society on Thrombosis and Haemostasis

A key role for JNK in mediating the differential augmenting action of histamine on TF expression is underscored by the effect of histamine on VCAM-1 expression. In contrast to TF, histamine enhanced TNF-α- and thrombin-induced VCAM-1 expression to a similar extent. Analysis of the signal transduction pathways revealed that p38 mediates VCAM-1 induction in response to TNF-α and thrombin. In contrast, inhibition of JNK did not result in a reduced VCAM-1 expression in response to TNF-α or thrombin, both in the presence and the absence of histamine; hence, JNK does not seem to regulate endothelial VCAM-1 induction. Consistent with this observation, no differential augmentation of histamine was observed with TNF-α- vs. thrombin-induced VCAM-1 expression. Other studies found that JNK is involved in TNF-α-induced VCAM-1 induction [27,28]: different cell culture protocols, different TNF-α concentrations, or higher concentrations of the JNK inhibitor in these studies may account for this difference. Overall, there is increasing evidence that JNK plays an important role in the development of vascular disease and atherosclerosis; JNK is indeed involved in a variety of proatherogenic cellular processes, including endothelial cell activation, foam cell formation, and vascular smooth muscle cell migration and proliferation [29].

TF is known to be involved in the pathogenesis of vascular disease [12,30], and elevated levels of TF have been observed in patients with hypertension, dyslipidemia, diabetic vasculopathy, and peripheral artery disease [12,31–34]. A pathogenetic role of histamine in atherogenesis was highlighted by recent studies demonstrating that histidine decarboxylase as well as the H_1-receptor are expressed in atherosclerotic human arteries [11], and that mice deficient in histidine decarboxylase exhibit reduced intimal thickening after vascular injury [10]. Invasion and activation of leukocytes are early events in atherogenesis. Histamine and TNF-α are released concomitantly by mast cells *in vivo* and exhibit a remarkable synergy regarding endothelial cell activation; indeed, histamine-induced endothelial interleukin production is enhanced by TNF-α [35,36], and TNF-α-induced expression of intercellular adhesion molecule-1 and E-selectin is potentiated by histamine [37]. Our study adds to the evidence that histamine and TNF-α effectively interact in endothelial activation and vascular inflammation and indicates that the potent augmentation of TNF-α-induced TF expression by histamine may play a role in the pathogenesis of thrombotic vascular disorders such as acute coronary syndromes or stroke [12]. Indeed, several studies observed an increase in mast cells and histamine in coronary arteries of patients with ischemic heart disease [9,38]. In addition, histamine elicits potent arterial contractions [21,39], which can lead to variant angina and have also been implicated in arterial thrombus formation [40–42]. The enhancing effect of histamine on TNF-α- and thrombin-induced TF expression may accelerate thrombus formation and propagation and, eventually, vessel occlusion.

In summary, histamine differentially augments TNF-α- vs. thrombin-induced TF expression; a remarkable synergy of histamine and TNF-α resulted in a strong potentiation of TF induction, while the amine only slightly enhanced thrombin-induced TF expression. Given the importance of vascular inflammation for TF expression and thrombus formation, these data have interesting implications for the pathogenesis of vascular disease. Because the potentiating effect of histamine on TNF-α- and thrombin-induced TF expression is abrogated by H_1-receptor antagonists, our findings further point to a therapeutic potential of these drugs in the treatment of clinical manifestations of atherosclerosis, such as acute coronary syndromes.

Acknowledgements

This study was supported by the Swiss National Science Foundation (grant # 3200B0-102232/1 to F. C. Tanner and grant # 3100-068118.02/1 to T. F. Lüscher), Wolfermann-Nägeli Foundation, Bonizzi-Theler Foundation, Hartmann Müller Foundation, Herzog-Egli Foundation, and Olga Mayenfisch Foundation.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Hollis TM, Gallik SG, Orlidge A, Yost JC. Aortic endothelial and smooth muscle histamine metabolism. Relationship to aortic 125I-albumin accumulation in experimental diabetes. *Arteriosclerosis* 1983; **3**: 599–606.
2 Brown MJ, Ind PW, Jenner DA. Platelet histamine. *N Engl J Med* 1980; **303**: 756.
3 Mannaioni PF, Di Bello MG, Masini E. Platelets and inflammation: role of platelet-derived growth factor, adhesion molecules and histamine. *Inflamm Res* 1997; **46**: 4–18.
4 Ross R. Atherosclerosis – an inflammatory disease. *N Engl J Med* 1999; **340**: 115–26.
5 Libby P. Inflammation in atherosclerosis. *Nature* 2002; **420**: 868–74.
6 Lee M, Sommerhoff CP, von Eckardstein A, Zettl F, Fritz H, Kovanen PT. Mast cell tryptase degrades HDL and blocks its function as an acceptor of cellular cholesterol. *Arterioscler Thromb Vasc Biol* 2002; **22**: 2086–91.
7 Atkinson JB, Harlan CW, Harlan GC, Virmani R. The association of mast cells and atherosclerosis: a morphologic study of early atherosclerotic lesions in young people. *Hum Pathol* 1994; **25**: 154–9.
8 Kaartinen M, van der Wal AC, van der Loos CM, Piek JJ, Koch KT, Becker AE, Kovanen PT. Mast cell infiltration in acute coronary syndromes: implications for plaque rupture. *J Am Coll Cardiol* 1998; **32**: 606–12.
9 Laine P, Kaartinen M, Penttila A, Panula P, Paavonen T, Kovanen PT. Association between myocardial infarction and the mast cells in the adventitia of the infarct-related coronary artery. *Circulation* 1999; **99**: 361–9.
10 Sasaguri Y, Wang KY, Tanimoto A, Tsutsui M, Ueno H, Murata Y, Kohno Y, Yamada S, Ohtsu H. Role of histamine produced by bone marrow-derived vascular cells in pathogenesis of atherosclerosis. *Circ Res* 2005; **96**: 974–81.
11 Murata Y, Tanimoto A, Wang KY, Tsutsui M, Sasaguri Y, De Corte F, Matsushita H. Granulocyte macrophage-colony stimulating factor

© 2006 International Society on Thrombosis and Haemostasis

increases the expression of histamine and histamine receptors in monocytes/macrophages in relation to arteriosclerosis. *Arterioscler Thromb Vasc Biol* 2005; **25**: 430–5.
12 Steffel J, Luscher TF, Tanner FC. Tissue factor in cardiovascular disease: molecular mechanisms and clinical implications. *Circulation* 2006; **113**: 722–31.
13 Steffel J, Akhmedov A, Greutert H, Luscher TF, Tanner FC. Histamine induces tissue factor expression: implications for acute coronary syndromes. *Circulation* 2005; **112**: 341–9.
14 Steffel J, Hermann M, Greutert H, Gay S, Luscher TF, Ruschitzka F, Tanner FC. Celecoxib decreases endothelial tissue factor expression through inhibition of c-Jun terminal NH2 kinase phosphorylation. *Circulation* 2005; **111**: 1685–9.
15 Tanner FC, Meier P, Greutert H, Champion C, Nabel EG, Luscher TF. Nitric oxide modulates expression of cell cycle regulatory proteins: a cytostatic strategy for inhibition of human vascular smooth muscle cell proliferation. *Circulation* 2000; **101**: 1982–9.
16 Bogdanov VY, Balasubramanian V, Hathcock J, Vele O, Lieb M, Nemerson Y. Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. *Nat Med* 2003; **9**: 458–62.
17 Cui MZ, Zhao G, Winokur AL, Laag E, Bydash JR, Penn MS, Chisolm GM, Xu X. Lysophosphatidic acid induction of tissue factor expression in aortic smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2003; **23**: 224–30.
18 Liu Y, Pelekanakis K, Woolkalis MJ. Thrombin and TNFalpha synergistically stimulate tissue factor expression in human endothelial cells: regulation through c-Fos and c-Jun. *J Biol Chem* 2004; **279**: 36142–7.
19 Steffel J, Latini RA, Akhmedov A, Zimmermann D, Zimmerling P, Luscher TF, Tanner FC. Rapamycin, but not FK-506, increases endothelial tissue factor expression: implications for drug-eluting stent design. *Circulation* 2005; **112**: 2002–11.
20 Steffel J, Akhmedov A, Fahndrich C, Ruschitzka F, Luscher TF, Tanner FC. Differential effect of celecoxib on tissue factor expression in human endothelial and vascular smooth muscle cells. *Biochem Biophys Res Commun* 2006; **349**: 597–603.
21 Shimokawa H, Tomoike H, Nabeyama S, Yamamoto H, Araki H, Nakamura M, Ishii Y, Tanaka K. Coronary artery spasm induced in atherosclerotic miniature swine. *Science* 1983; **221**: 560–2.
22 Asako H, Kurose I, Wolf R, DeFrees S, Zheng ZL, Phillips ML, Paulson JC, Granger DN. Role of H1 receptors and P-selectin in histamine-induced leukocyte rolling and adhesion in postcapillary venules. *J Clin Invest* 1994; **93**: 1508–15.
23 Miyazawa N, Watanabe S, Matsuda A, Kondo K, Hashimoto H, Umemura K, Nakashima M. Role of histamine H1 and H2 receptor antagonists in the prevention of intimal thickening. *Eur J Pharmacol* 1998; **362**: 53–59.
24 Bartha K, Brisson C, Archipoff G, de la Salle C, Lanza F, Cazenave JP, Beretz A. Thrombin regulates tissue factor and thrombomodulin mRNA levels and activities in human saphenous vein endothelial cells by distinct mechanisms. *J Biol Chem* 1993; **268**: 421–9.
25 Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J Biol Chem* 2002; **277**: 32124–32.
26 Eto M, Kozai T, Cosentino F, Joch H, Luscher TF. Statin prevents tissue factor expression in human endothelial cells: role of Rho/Rho-kinase and Akt pathways. *Circulation* 2002; **105**: 1756–9.
27 Lin SJ, Shyue SK, Hung YY, Chen YH, Ku HH, Chen JW, Tam KB, Chen YL. Superoxide dismutase inhibits the expression of vascular cell adhesion molecule-1 and intracellular cell adhesion molecule-1 induced by tumor necrosis factor-alpha in human endothelial cells through the JNK/p38 pathways. *Arterioscler Thromb Vasc Biol* 2005; **25**: 334–40.
28 Yoshizumi M, Fujita Y, Izawa Y, Suzaki Y, Kyaw M, Ali N, Tsuchiya K, Kagami S, Yano S, Sone S, Tamaki T. Ebselen inhibits tumor necrosis factor-alpha-induced c-Jun N-terminal kinase activation and adhesion molecule expression in endothelial cells. *Exp Cell Res* 2004; **292**: 1–10.
29 Manning AM, Davis RJ. Targeting JNK for therapeutic benefit: from junk to gold? *Nat Rev Drug Discov* 2003; **2**: 554–65.
30 Tremoli E, Camera M, Toschi V, Colli S. Tissue factor in atherosclerosis. *Atherosclerosis* 1999; **144**: 273–83.
31 Felmeden DC, Spencer CG, Chung NA, Belgore FM, Blann AD, Beevers DG, Lip GY. Relation of thrombogenesis in systemic hypertension to angiogenesis and endothelial damage/dysfunction (a substudy of the Anglo-Scandinavian Cardiac Outcomes Trial [ASCOT]). *Am J Cardiol* 2003; **92**: 400–5.
32 Blann AD, Amiral J, McCollum CN, Lip GY. Differences in free and total tissue factor pathway inhibitor, and tissue factor in peripheral artery disease compared to healthy controls. *Atherosclerosis* 2000; **152**: 29–34.
33 Lim HS, Blann AD, Lip GY. Soluble CD40 ligand, soluble P-selectin, interleukin-6, and tissue factor in diabetes mellitus: relationships to cardiovascular disease and risk factor intervention. *Circulation* 2004; **109**: 2524–8.
34 Sambola A, Osende J, Hathcock J, Degen M, Nemerson Y, Fuster V, Crandall J, Badimon JJ. Role of risk factors in the modulation of tissue factor activity and blood thrombogenicity. *Circulation* 2003; **107**: 973–7.
35 Li Y, Chi L, Stechschulte DJ, Dileepan KN. Histamine-induced production of interleukin-6 and interleukin-8 by human coronary artery endothelial cells is enhanced by endotoxin and tumor necrosis factor-alpha. *Microvasc Res* 2001; **61**: 253–62.
36 Jeannin P, Delneste Y, Gosset P, Molet S, Lassalle P, Hamid Q, Tsicopoulos A, Tonnel AB. Histamine induces interleukin-8 secretion by endothelial cells. *Blood* 1994; **84**: 2229–33.
37 Miki I, Kusano A, Ohta S, Hanai N, Otoshi M, Masaki S, Sato S, Ohmori K. Histamine enhanced the TNF-alpha-induced expression of E-selectin and ICAM-1 on vascular endothelial cells. *Cell Immunol* 1996; **171**: 285–8.
38 Kalsner S, Richards R. Coronary arteries of cardiac patients are hyperreactive and contain stores of amines: a mechanism for coronary spasm. *Science* 1984; **223**: 1435–7.
39 Stahli BE, Greutert H, Shaoping M, Graf P, Frischknecht K, Stalder M, Englberger L, Künzli A, Schärer L, Luscher TF, Carrel TP, Tanner FC. Absence of histamine-induced nitric oxide release in the human radial artery: implications for vasospasm of coronary artery bypass vessels. *Am J Physiol – Heart* 2006; **290**: 1182–9.
40 Oliva PB, Breckinridge JC. Arteriographic evidence of coronary arterial spasm in acute myocardial infarction. *Circulation* 1977; **56**: 366–74.
41 Buja LM, Hillis LD, Petty CS, Willerson JT. The role of coronary arterial spasm in ischemic heart disease. *Arch Pathol Lab Med* 1981; **105**: 221–6.
42 Hackett D, Davies G, Chierchia S, Maseri A. Intermittent coronary occlusion in acute myocardial infarction. Value of combined thrombolytic and vasodilator therapy. *N Engl J Med* 1987; **317**: 1055–9.

© 2006 International Society on Thrombosis and Haemostasis

Rapamycin, but Not FK-506, Increases Endothelial Tissue Factor Expression

Implications for Drug-Eluting Stent Design

Jan Steffel, MD*; Roberto A. Latini, MD*; Alexander Akhmedov, PhD; Dorothee Zimmermann, BSc; Pamela Zimmerling, BSc; Thomas F. Lüscher, MD; Felix C. Tanner, MD

Background—Drugs released from stents affect the biology of vascular cells. We examined the effect of rapamycin and FK-506 on tissue factor (TF) expression in human aortic endothelial cells (HAECs) and vascular smooth muscle cells (HAVSMCs).

Methods and Results—Rapamycin enhanced thrombin- and tumor necrosis factor (TNF)-α–induced endothelial TF expression in a concentration-dependent manner. The maximal increase was 2.5-fold more pronounced than that by thrombin or TNF-α alone and was paralleled by a 1.4-fold higher TF surface activity compared with thrombin alone. Rapamycin by itself increased basal TF levels by 40%. In HAVSMCs, rapamycin did not affect thrombin- or TNF-α–induced TF expression. In contrast to rapamycin, FK-506 did not enhance thrombin- or TNF-α–induced endothelial TF expression. Thrombin induced a transient dephosphorylation of the mammalian target of rapamycin downstream target p70S6 kinase. Rapamycin completely abrogated p70S6 kinase phosphorylation, but FK-506 did not. FK-506 antagonized the effect of rapamycin on thrombin-induced TF expression. Rapamycin did not alter the pattern of p38, extracellular signal–regulated kinase, or c-Jun NH_2-terminal kinase phosphorylation. Real-time polymerase chain reaction analysis revealed that rapamycin had no influence on thrombin-induced TF mRNA levels for up to 2 hours but led to an additional increase after 3 and 5 hours.

Conclusions—Rapamycin, but not FK-506, enhances TF expression in HAECs but not in HAVSMCs. This effect requires binding to FK binding protein-12, is mediated through inhibition of the mammalian target of rapamycin, and partly occurs at the posttranscriptional level. These findings may be clinically relevant for patients receiving drug-eluting stents, particularly when antithrombotic drugs are withdrawn or ineffective, and may open novel perspectives for the design of such stents. **(*Circulation*. 2005;112:2002-2011.)**

Key Words: endothelium ■ myocardial infarction ■ signal transduction ■ stents ■ thrombosis

Percutaneous coronary intervention with stenting of the culprit lesion is the preferred treatment for patients with acute coronary syndromes.[1–3] Several clinical trials have demonstrated that drug-eluting stents (DESs) are superior to bare-metal stents (BMSs) by decreasing the restenosis rates as well as major adverse cardiac events.[4–6] Rapamycin (sirolimus), a macrocyclic lactone, is used on DESs because the drug inhibits proliferation and migration of vascular smooth muscle cells (VSMCs).[7] FK-506 (tacrolimus), a macrolide immunosuppressant, is an alternative drug used with DESs.[8,9] Despite reduced restenosis rates, however, stent thromboses have not decreased with DESs compared with BMSs.[6,10–12] Indeed, several hundred cases of in-stent thrombosis have been reported with rapamycin-coated stents,[13] and results from a recent multicenter registry imply that thrombosis rates with DESs may be higher in "real world" patients than reported in previous clinical trials.[14] The reason for the discrepancy between reduced restenosis rates and unaltered or even enhanced thrombosis rates with DESs compared with BMSs is not known.[6,12]

Several factors are involved in the pathogenesis of in-stent thrombosis. These include procedure-related factors such as mechanical vessel injury or incomplete stent apposition, patient-related factors such as vessel size or coagulation activity, and finally, the thrombogenicity of the stent itself.[15] It has not yet been explored, however, whether the drugs used for stent coating could be involved in the development of in-stent thrombosis.[15]

Tissue factor (TF), a 263-residue, membrane-bound glycoprotein, is a key enzyme in the initiation of coagulation; it

Received January 7, 2005; de novo received June 15, 2005; accepted July 8, 2005.

From Cardiovascular Research, Physiology Institute, and the Center for Integrative Human Physiology (J.S., R.A.L., A.A., D.Z., P.Z., T.F.L., F.C.T.), University of Zurich, and the Department of Cardiology (J.S., T.F.L., F.C.T.), Cardiovascular Center, University Hospital Zürich, Zürich, Switzerland.

*The first 2 authors contributed equally to the study.

Guest Editor for this article was James T. Willerson, MD.

Correspondence to Felix C. Tanner, MD, Cardiovascular Research, Physiology Institute, University of Zurich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. E-mail felix.tanner@access.unizh.ch

© 2005 American Heart Association, Inc.

Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/CIRCULATIONAHA.105.569129**

activates factor X (FX) by binding activated factor VII (FVIIa), which ultimately leads to thrombin formation. Initiation of coagulation is a key event in the pathogenesis of thrombosis and acute coronary syndromes. Not surprisingly, atheromatous plaques contain a variety of cells expressing TF, including endothelial cells (ECs) and VSMCs. Moreover, TF levels are elevated in the plasma and atherectomy samples from patients with unstable angina.[16] Therefore, TF seems to be involved in the development of atherosclerosis and restenosis after percutaneous coronary intervention.[17–19] TF may indeed play a major role in stent thrombosis as well. However, the effect of neither rapamycin nor FK-506 on TF expression has been investigated so far. Moreover, the role of the mammalian target of rapamycin (mTOR) in regulating TF expression is also not known. Thus, the present study was designed to investigate the influence of rapamycin and FK-506 on TF expression in human aortic endothelial cells (HAECs) and vascular smooth muscle cells (HAVSMCs).

Methods

Cell Culture

HAECs and HAVSMCs were cultured as described.[20,21] Cells were grown to confluence in 6-cm culture dishes and rendered quiescent for 24 hours before stimulation with thrombin or tumor necrosis factor (TNF)-α (Sigma). Rapamycin, wortmannin (both from Sigma), FK-506 (Alexis), and LY294002 (Cell Signaling) were added to the dishes 60 minutes before stimulation. Cytotoxicity was assessed with a colorimetric assay to detect lactate dehydrogenase release according to the manufacturer's recommendations (Roche).

Western Blot Analysis and ELISA

Protein expression was determined by Western blot analysis as described.[22,23] Cells were lysed in 50 mmol/L Tris buffer, and 30-μg samples were loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) by semidry transfer. Antibody to human TF (American Diagnostica) was used at 1:2000 dilution; antibodies against the phosphorylated Thr-389 residue of p70S6 kinase (S6K), phosphorylated p38 mitogen-activated protein (MAP) kinase (p38), phosphorylated p44/42 MAP kinase (extracellular signal–regulated kinase [ERK]), and phosphorylated c-Jun NH_2-terminal kinase (JNK; all from Cell Signaling) were used at 1:3000, 1:1000, 1:5000, and 1:1000 dilution, respectively. Antibodies against total S6K, total p38, total ERK, and total JNK (all from Cell Signaling) were used at 1:3000, 1:2000, 1:10 000, and 1:1000 dilution, respectively. All blots were normalized to α-tubulin (aT) expression (1:20 000 dilution, Sigma). Endothelial TF expression was also measured with a commercially available ELISA (American Diagnostica) according to the supplier's recommendations.

Real-Time PCR Analysis

RNA was extracted and converted to cDNA as described.[22] Real-time polymerase chain reaction (PCR) was performed in an MX3000P PCR cycler (Stratagene). All PCR experiments were performed with the SYBR Green JumpStart kit (Sigma). Each reaction (25 μL) contained 2 μL cDNA, 1 pmol of each primer, 0.25 μL of internal reference dye, and 12.5 μL of JumpStart *Taq* ReadyMix (containing buffer, dNTPs, stabilizers, SYBR Green, *Taq* polymerase, and JumpStart *Taq* antibody). Primers for human TF were used as described.[22,24] Expression of the ribosomal protein L28 (L28) mRNA was used as a loading control; primers for human L28 were designed as follows: sense primer, 5′-GCATCTGC-AATGGATGGT-3′ and antisense primer, 5′-TGTTCTTGCG-GATCATGTGT-3′. The amplification program consisted of 1 cycle at 95°C for 10 minutes; followed by 40 cycles with a denaturing phase at 95°C for 30 seconds, an annealing phase at 60°C for 1 minute, and an elongation phase at 72°C for 1 minute. A melting curve analysis was performed after amplification to verify the homogeneity of the amplicon. For verification of amplicon size, PCR products were analyzed on an ethidium bromide–stained 1% agarose gel. In each real-time PCR run for TF and L28, a calibration curve generated from serial dilutions of a known TF and L28 standard, respectively, was included, and for each sample, the target values were corrected by those for L28.

TF Surface Activity

A colorimetric assay (American Diagnostica) was used to analyze TF surface activity according to the manufacturer's recommendations, with some modifications as described.[22,25] Cells were grown in 6-well plates; after stimulation, cells were washed twice with phosphate-buffered saline and incubated with human FVIIa and FX at 37°C, resulting in the formation of a TF/FVIIa complex at the cell surface. The TF/FVIIa complex converted human FX to FXa, which was subsequently measured by its ability to cleave a chromogenic substrate. Different concentrations of lipidated human TF were used as positive controls to confirm that the obtained results were in the linear range of detection (data not shown).

Proliferation

To examine the effect of rapamycin and FK-506 on EC proliferation, HAECs were seeded on 6-cm dishes at 7000 cells/cm^2. After 24 hours, when cells had reached ≈50% confluence, they were serum-starved for 24 hours before incubation with rapamycin (10^{-7} mol/L), FK-506 (10^{-7} mol/L), or carrier (0.1% dimethyl sulfoxide) in endothelial basal medium (EBM, Clonetics) containing 10% fetal calf serum (FCS). At the indicated times, cells were gently trypsinized and counted in a hemacytometer. Each analysis was performed in duplicate; results are representative of 3 independent experiments.

Apoptosis

To assess induction of apoptosis by rapamycin and FK-506, cells were cultured in chamber slides (Nunc) at 20 000 cells/well for 24 hours before serum-starvation for 24 hours. Cells were then incubated in EBM with 10% FCS containing rapamycin (10^{-7} mol/L), FK-506 (10^{-7} mol/L), or carrier (0.1% dimethyl sulfoxide). At the indicated times, cells were fixed with 4% paraformaldehyde and processed for terminal deoxynucleotidyl nick end-labeling (TUNEL) staining with a commercially available kit (Roche) according to the manufacturer's recommendations. Afterward, cells were counterstained with 4′,6 diamidino-2-phenylindole (DAPI; Vector) and counted under a fluorescence microscope. Two hundred cells per time point and condition were counted, and the number of TUNEL-positive cells was assessed.

Statistics

Data are presented as mean±SEM. Unpaired Student *t* test was used for statistical analysis. A probability value <0.05 was considered significant.

Results

Rapamycin Enhances TF Expression in HAECs but Not HAVSMCs

Stimulation of HAECs with thrombin (1 U/mL) induced TF expression 23-fold as assessed by Western blotting analysis (Figure 1A). Incubation with rapamycin (10^{-8} to 10^{-7} mol/L) before stimulation with thrombin resulted in a concentration-dependent enhancement of TF expression (Figure 1A). The maximal increase was observed after 5 hours and was 2.3-fold compared with stimulation with thrombin alone and 51-fold compared with the basal level. Similarly, rapamycin (10^{-8} to 10^{-7} mol/L) enhanced TF expression in response to TNF-α (5 ng/mL); this increase was 2.5-fold, resulting in a

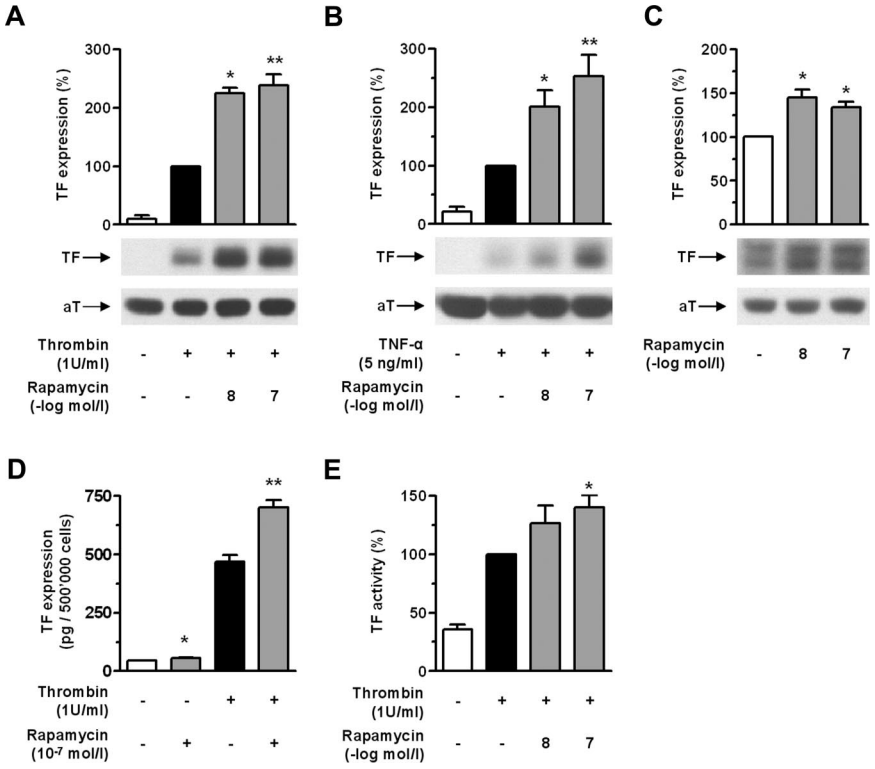


Figure 1. Rapamycin enhances TF expression in HAECs. A, Rapamycin enhances thrombin-induced TF expression in a concentration-dependent manner. Values are given as a percentage of stimulation with thrombin alone. *$P<0.0001$, ** $P<0.001$ vs thrombin alone. B, Rapamycin enhances TNF-α–induced TF expression in a concentration-dependent manner. Values are given as a percentage of stimulation with TNF-α alone. *$P<0.02$, ** $P<0.01$ vs TNF-α alone. C, Rapamycin increases basal TF expression. Values are given as a percentage of unstimulated control. *$P<0.01$ vs unstimulated control. Values are representative of at least 3 different experiments; all blots were normalized to aT expression. D, TF ELISA confirms that rapamycin increases both basal (*$P<0.05$ vs unstimulated control) and thrombin-induced (**$P<0.01$ vs thrombin alone) TF expression. E, Rapamycin enhances thrombin-induced TF surface activity in a concentration-dependent manner. Values are given as a percentage of stimulation with thrombin alone. *$P<0.01$ vs thrombin alone.

35-fold induction compared with the basal level (Figure 1B). ECs express TF only at very low levels under basal conditions,[26] and stimulation of HAECs with rapamycin alone increased basal TF expression by 40%, as assessed by Western blotting analysis (Figure 1C), or 25% as assessed by ELISA (Figure 1D). Expression of TF was 45±1 pg per 500 000 cells for control, 57±3 pg per 500′000 cells for rapamycin (10^{-7} mol/L) alone, 468±29 pg per 500 000 cells for thrombin stimulation, and 702±30 pg/500 000 cells for thrombin stimulation in the presence of rapamycin (Figure 1D). The rapamycin-enhanced increase in thrombin-induced TF expression was paralleled by an increase of TF surface activity, which reached 1.4 times the level induced by thrombin alone (Figure 1E).

Similar to HAECs, thrombin (1 U/mL) and TNF-α (5 ng/mL) induced TF expression in HAVSMCs. In contrast to HAECs, however, rapamycin did not affect TF expression in response to either mediator in this cell type (Figure 2A and 2B). No cytotoxic effect of rapamycin was observed for any of the concentrations used (n=4, P=NS; data not shown).

FK-506 Does Not Affect TF Expression

Incubation with FK-506 (10^{-8} to 10^{-7} mol/L) before stimulation with thrombin (1 U/mL, Figure 3A) or TNF-α (5 ng/mL, Figure 3B) did not alter TF expression. No cytotoxic effect of FK-506 was observed for any of the concentrations used (n=4, P=NS; data not shown).

Journal of the American Heart Association

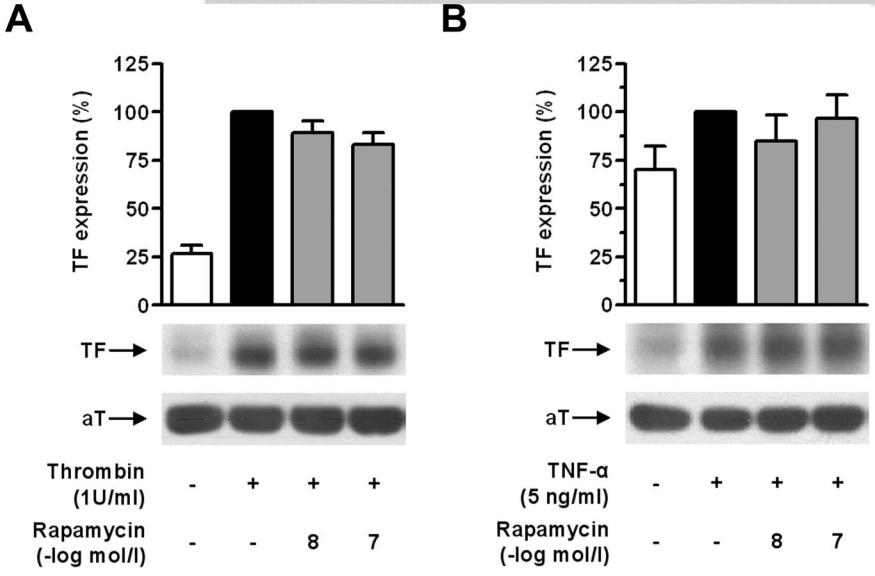


Figure 2. Rapamycin does not affect TF expression in HAVSMCs. Rapamycin does not affect thrombin- (A) or TNF-α–(B) induced TF expression in HAVSMCs. Values are given as a percentage of stimulation with thrombin (1 U/mL) or TNF-α (5 ng/mL) alone. Blots are representative of at least 4 different experiments; all blots were normalized to aT expression.

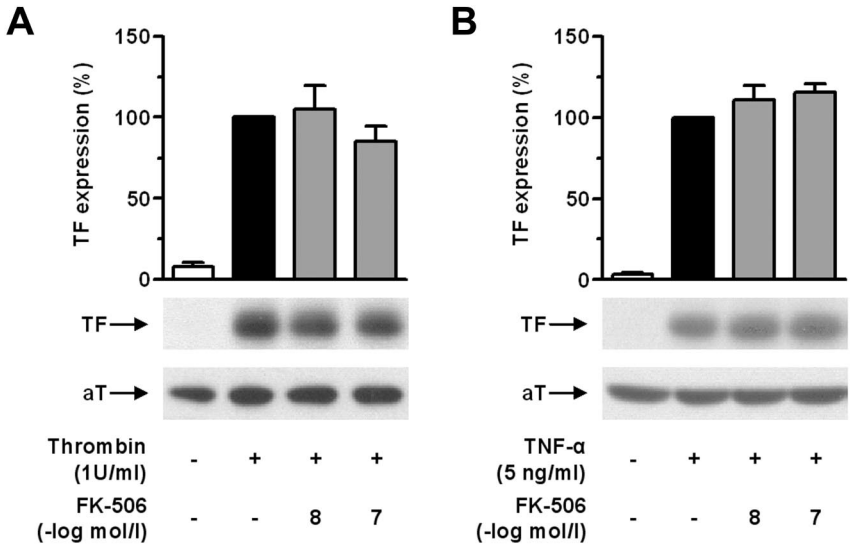


Figure 3. FK-506 does not affect TF expression in HAECs. FK-506 does not affect thrombin- (A) or TNF-α– (B) induced TF expression in HAECs. Values are presented as a percentage of stimulation with thrombin (1 U/mL) or TNF-α (5 ng/mL) alone. Blots are representative of at least 3 different experiments; all blots were normalized to aT expression.

Rapamycin Enhances TF Expression by Inhibiting mTOR Activity

Phosphorylation of S6K, a downstream target of the mTOR, is frequently used to assess mTOR inhibition by rapamycin.[27,28] When stimulated with thrombin (1 U/mL), S6K phosphorylation was transiently decreased after 30 minutes to a minimum of 19% of the basal level (Figure 4A, left). Rapamycin (10^{-7} mol/L) completely abrogated S6K phosphorylation, in both the presence and absence of thrombin (Figure 4A, right). Similarly, inhibition of phosphatidyl inositol 3-kinase with LY294002 or wortmannin almost completely abrogated S6K phosphorylation, again independent of thrombin stimulation (Figure 4C). In contrast, FK-506 (10^{-7} mol/L) did not affect phosphorylation of S6K in either the presence or absence of thrombin (Figure 4B).

Rapamycin Enhances TF Expression by Binding to FKBP-12

Rapamycin and FK-506 bind to the same intracellular receptor, FK binding protein-12 (FKBP-12). When HAECs were treated with increasing concentrations of FK-506 for 30 minutes before incubation with rapamycin, FK-506 reduced the effect of rapamycin on thrombin-induced TF expression (Figure 5). Indeed, when incubated with the highest concentration of FK-506 (10^{-7} mol/L), the increase in TF expression elicited by rapamycin with respect to stimulation with thrombin alone was reduced by 41% ($P<0.05$).

Effect of Rapamycin on Thrombin-Induced TF mRNA Levels

Real-time PCR revealed that thrombin induced TF mRNA expression in a time-dependent manner (Figure 6A). Rapamycin did not alter thrombin-induced mRNA expression compared with stimulation by thrombin alone after 0.5, 1, and 2 hours. However, after 3 and 5 hours of stimulation, rapamycin significantly augmented thrombin-induced TF mRNA levels (Figure 6A and 6B). Rapamycin significantly increased thrombin-induced TF protein expression after 3, 5, and 7 hours compared with stimulation by thrombin alone (Figure 6C).

Rapamycin did not affect the pattern of MAP kinase activation observed after thrombin stimulation. Indeed, phosphorylation of p38 (Figure 7A), ERK (Figure 7B), and JNK (Figure 7C) remained unaltered after pretreatment with rapamycin compared with stimulation by thrombin alone.

Rapamycin, but Not FK-506, Inhibits EC Proliferation

EC proliferation was induced by incubation with EBM containing 10% FCS (Figure 8A, control). Rapamycin (10^{-7} mol/L) prevented FCS-induced EC proliferation. In contrast, FK-506 (10^{-7} mol/L) did not significantly inhibit EC proliferation (Figure 8A).

TUNEL staining was used to examine whether rapamycin (10^{-7} mol/L) or FK-506 (10^{-7} mol/L) induced apoptosis in HAECs (Figure 8B). Representative sections are shown. After 24 hours, TUNEL-positive cells accounted for 5.3±0.7% of cells in the control group, 4.7±1.7% for rapamycin (P=NS versus control), and 4.8±1.5% for FK-506 (P=NS versus control). After 48 hours, 4.2±1.7% of control cells, 3.5±0.6% of rapamycin-treated cells (P=NS versus control), and 4.6±2.3% of FK-506-treated cells (P=NS versus control) were TUNEL-positive. Cells incubated with H_2O_2 (1 mmol/L) for 6 hours as well as serum withdrawal for 48 hours served as positive controls and resulted in a significant increase in apoptotic cells (data not shown). Thus, neither rapamycin (10^{-7} mol/L) nor FK-506 (10^{-7} mol/L) led to an increase in apoptotic cells compared with control conditions.

Discussion

This study demonstrates that rapamycin enhances endothelial TF expression in response to thrombin and TNF-α. The concentrations of rapamycin occurring in vivo compare well with those used in our study, as maximal systemic concentrations of rapamycin after deployment of 2 sirolimus-eluting

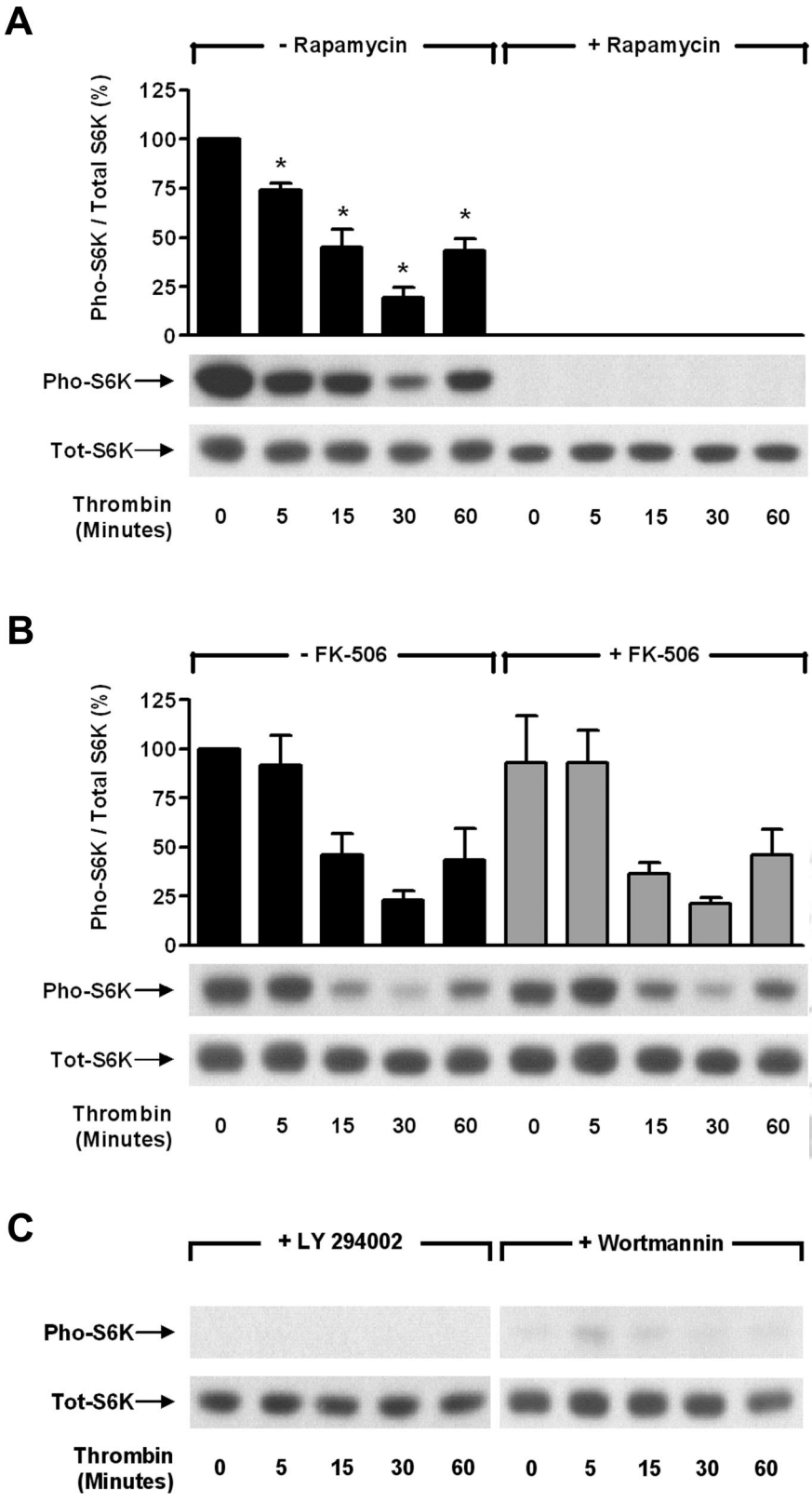


Figure 4. Rapamycin inhibits endothelial mTOR activity. A, Thrombin leads to a transient, time-dependent inhibition of S6K phosphorylation (left). Rapamycin (right) completely abrogates S6K phosphorylation in both the presence and absence of thrombin. Total levels of S6K remain unchanged. Values are presented as phosphorylated (Pho) S6K/total (Tot) S6K. *$P<0.0001$ vs unstimulated conditions. B, FK-506 affects neither basal phosphorylation levels nor thrombin-induced inhibition of S6K phosphorylation. Values are presented as phosphorylated S6K (Pho)/total (Tot) S6K. C, LY294002 (5×10^{-6} mol/L, left) and wortmannin (10^{-7} mol/L, right) almost completely abrogate S6K phosphorylation (Pho). Total (Tot) levels of S6K remain unchanged.

stents are reported to be ≈1 ng/mL (≈1.15×10^{-9} mol/L)[29]; moreover, local concentrations, though difficult to assess, are likely to be significantly higher, partly because of rapamycin's lipophilic properties, leading to accumulation of the drug in the vessel wall.[11,30–32] Thus, the concentrations used in our study may be relevant for patients treated with DESs.

Reendothelialization is initiated soon after vascular injury; indeed, it has been observed to begin as early as 2 days after balloon dilation in animal models.[33–35] In humans, partial reendothelialization has been documented 3 weeks after stent deployment.[35–37] Sirolimus-eluting stents are designed in such a way that ≈80% of the rapamycin has eluted by 30

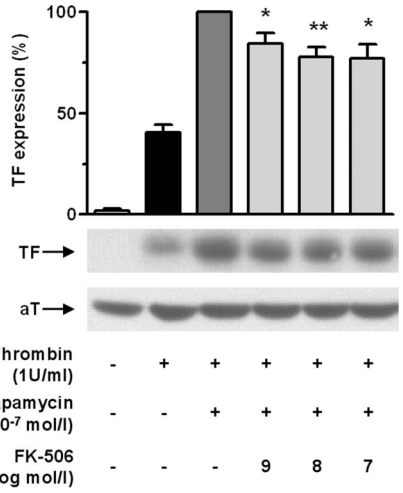


Figure 5. FK-506 antagonizes rapamycin-induced TF expression. Preincubation with FK-506 reduces rapamycin-enhanced TF expression. Values are presented as a percentage of stimulation with thrombin (1 U/mL) and rapamycin (10^{-7} mol/L). *$P<0.05$ and **$P<0.02$, compared with thrombin and rapamycin (10^{-7} mol/L). Blots are representative of at least 3 different experiments; all blots were normalized to aT expression.

days.[4,5] Furthermore, rapamycin easily penetrates cell walls owing to its lipophilic properties, leading to chronic retention of the drug in arterial tissue.[30–32] Thus, the time course of reendothelialization versus the kinetics of rapamycin release suggests that rapamycin-enhanced endothelial TF expression may be involved in the pathogenesis of in-stent thrombosis. In addition, inhibition of endothelial proliferation by rapamycin indicates that rapamycin delays reendothelialization, which may increase stent thrombogenicity even further.

Several hundred cases of acute and subacute in-stent thrombosis have been observed after deployment of rapamycin-eluting stents.[13] In addition and in contrast to BMSs, late thrombosis has been reported after withdrawal of antithrombotic drugs with DESs.[10] Most of these data originated from case reports or were collected in controlled clinical trials. Recent results from a large-scale, multicenter registry, however, indicate that in-stent thrombosis is likely underestimated under these circumstances and that it may occur at substantially higher rates in real world patients.[14] The pathogenesis of in-stent thrombosis has not yet been fully explored[15]; moreover, it is not known whether the pathogenic events leading to thromboses of DESs are similar to those of BMSs. Enhanced TF expression in the presence of rapamycin may indeed favor the development of in-stent thrombosis after deployment of sirolimus-eluting stents, particularly when clopidogrel is withdrawn or ineffective because of drug resistance.[38] FK-506, which neither affects endothelial TF expression nor inhibits EC proliferation, may provide a more favorable environment for reducing thromboses of DESs. To assess the implications of these findings in vivo, however, further studies are needed to examine the degree as well as the spatiotemporal pattern of TF expression in the arterial wall after deployment of DESs.

Platelet activation is a crucial event in the pathogenesis of thrombus formation. Consequently, the use of platelet receptor blockers such as clopidogrel have greatly reduced the incidence of stent thromboses, whereas withdrawal of antiplatelet therapy favors thrombus formation.[10,14] Moreover, clopidogrel inhibits the release of TF from aggregating platelets,[39] which is of particular interest, as platelet aggregation and secretion are increased in human platelets treated with rapamycin.[40] Thus, effective antiplatelet therapy may account for the fact that thrombosis rates of sirolimus-eluting stents are not clearly higher than those of BMSs.

TF induction after deployment of rapamycin-eluting stents may also have a prothrombotic effect on ECs distal to the stented site. Indeed, remote effects of rapamycin have been demonstrated, with pronounced endothelial dysfunction in coronary arteries distal to sirolimus-eluting stents compared with BMSs.[41] Thus, in addition to the effect on ECs within the stented region, rapamycin may also increase TF expression in ECs in the distal coronary vasculature. Such an effect may also contribute to the no-reflow phenomenon after stent deployment.

Rapamycin did not enhance thrombin- or TNF-α–driven TF expression in HAVSMCs, indicating that rapamycin does not constitute an additional thrombogenic signal to the VSMC layer. Indeed, a much higher incidence of acute and subacute stent thromboses would be expected if rapamycin induced TF expression in VSMCs. Although the rapamycin-induced increase in endothelial TF expression may favor neointima formation via the release of growth factors from aggregating platelets, the inhibitory effect of rapamycin on the proliferation and migration of VSMC is very likely to protect the vessel from such effects.[7] Consistent with this interpretation, sirolimus-eluting stents reduce neointima formation despite inducing a procoagulative state owing to enhanced endothelial TF expression.

Both thrombin, a coagulation factor, and TNF-α, an inflammatory cytokine, are classic inducers of TF expression in vascular cells. Thrombin induced TF expression 27-fold when examined by Western blotting analysis and 10.3-fold by ELISA; similarly, rapamycin enhanced thrombin-induced TF expression by 2.3-fold in Western blot analysis and 1.5-fold by ELISA. This difference may be due to a different sensitivity and/or specificity of the 2 assays. In our study, rapamycin enhanced TF expression in response to both thrombin and TNF-α; it may thus upregulate TF expression in a prothrombotic as well as an inflammatory environment, both of which are encountered in the coronary vasculature after stent deployment.

Biologically active TF is located at the cell surface, and rapamycin-enhanced TF protein expression was indeed paralleled by an increase in TF surface activity. The increase in activity was not as pronounced as that of protein expression; this discrepancy has also been observed in response to thrombin alone.[42] The distribution of TF in several cellular compartments and/or the expression of encrypted TF might account for this difference.[43]

The inhibitory role of phosphatidyl inositol 3-kinase on TF expression is established, as its inhibition enhances TF expression in response to thrombin.[42,44] The mTOR is a downstream target of phosphatidyl inositol 3-kinase.[28] Binding of rapamycin to its intracellular receptor FKBP-12 leads

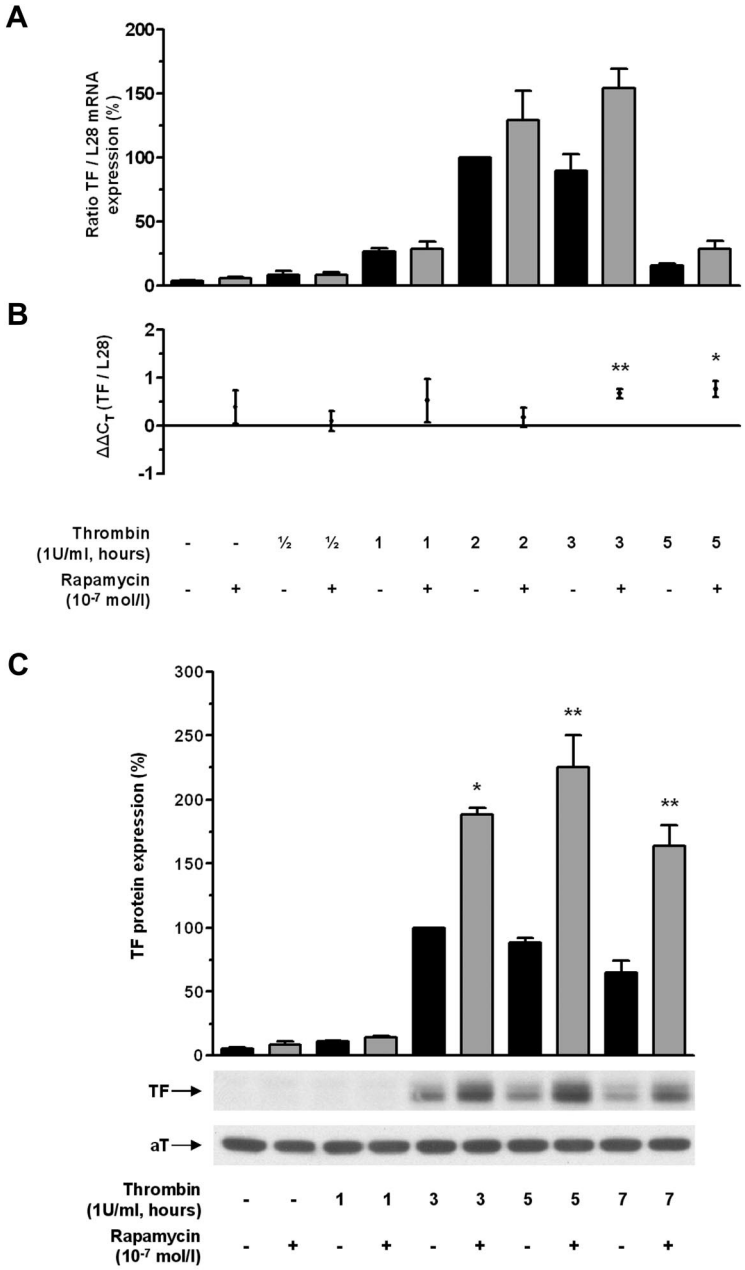


Figure 6. Effect of rapamycin on TF mRNA induction. A, Real-time PCR demonstrates a time-dependent induction of TF mRNA in response to thrombin. Rapamycin does not alter this pattern of induction after stimulation for 0.5, 1, and 2 hours. Values are given as a percentage of stimulation with thrombin alone for 2 hours. B, Analysis of $\Delta\Delta C_T$ values comparing the effect of rapamycin on thrombin-induced mRNA levels for every time point reveals that rapamycin significantly increases thrombin-induced TF mRNA after stimulation for 3 and 5 hours. $^*P<0.0005$, $^{**}P<0.005$. All values are representative of 4 different experiments and were normalized to L28 mRNA expression. C, Rapamycin enhances thrombin-induced TF protein expression in a time-dependent manner. Values are given as a percentage of stimulation with thrombin alone for 3 hours. $^*P<0.0001$, $^{**}P<0.01$ vs thrombin alone. Values are representative of at least 3 different experiments. All blots were normalized to aT expression.

American Heart Association. Fighting Heart Disease and Stroke

to formation of the rapamycin–FKBP-12 complex, which in turn inhibits mTOR activity. Phosphorylation of the downstream target of mTOR, S6K, is routinely used as a readout for the inhibitory effect of rapamycin on mTOR[27,28]; indeed, mTOR-dependent phosphorylation of the Thr-389 residue of S6K is necessary for its activity.[27] In the present study, we have shown that stimulation with thrombin leads to a transient inhibition of S6K phosphorylation. Rapamycin as well as the phosphatidyl inositol 3-kinase inhibitors wortmannin and LY294002 abrogated S6K phosphorylation in both the presence and absence of thrombin. Because thrombin stimulation as well as preincubation with rapamycin led to inhibition of this pathway, resulting in disinhibition of TF expression, these observations are consistent with the interpretation that mTOR plays an inhibitory role in TF expression.

FK-506 competitively binds to the same intracellular receptor as rapamycin, ie, FKBP-12.[45] In contrast to rapamycin, however, the FK-506–FKBP-12 complex inhibits the phosphatase calcineurin and has no effect on mTOR activity.[46] Consistently, FK-506 did not alter thrombin- or TNF-α–induced TF expression. To assess the specificity of our observations, we coincubated FK-506 and rapamycin before thrombin stimulation. The enhancing effect of rapamycin on thrombin-induced TF expression could indeed be reduced by FK-506. These findings indicate that binding of rapamycin to FKBP-12 is necessary for inhibition of mTOR activity and enhancement of TF expression.

TF expression in response to a variety of stimuli is mediated by MAP kinase activation, leading to increased transcription.[22,24,42] Indeed, thrombin induced an increase in p38, ERK, and JNK phosphorylation as well as an increase in TF transcription. However, rapamycin did not alter the pattern of thrombin-induced p38, ERK, and JNK activation.

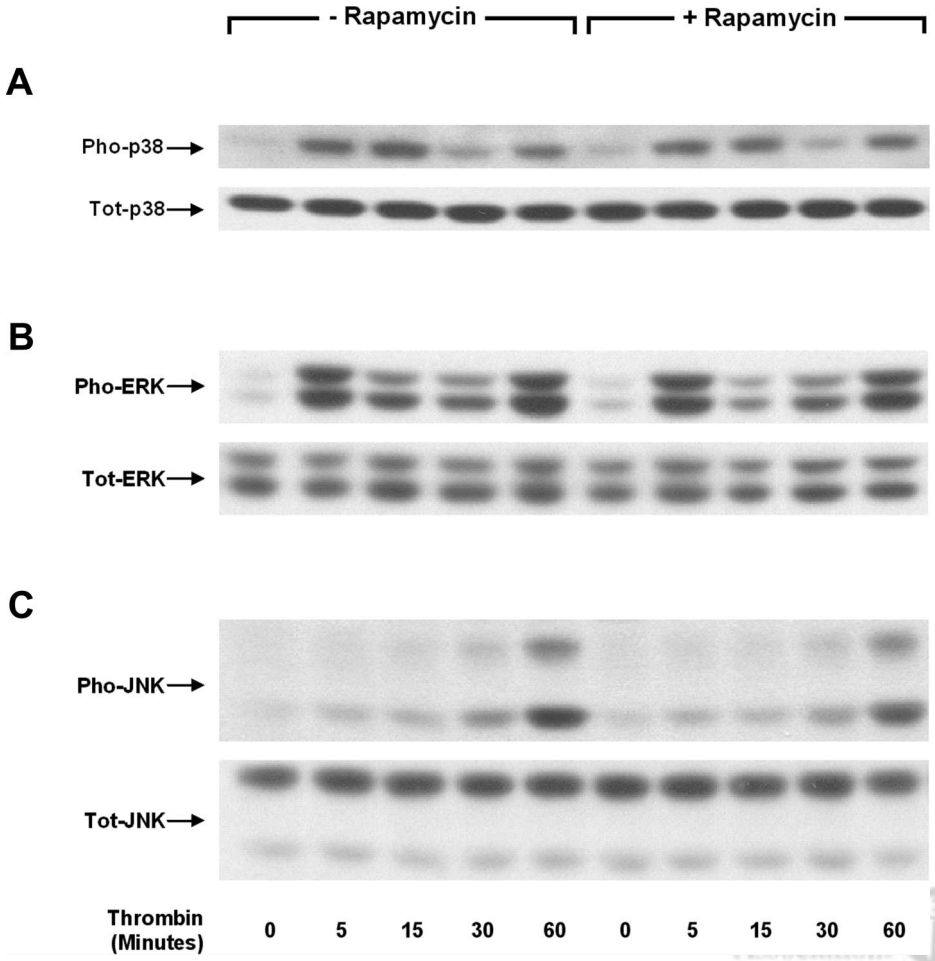


Figure 7. Rapamycin does not affect MAP kinase activation. Stimulation with thrombin leads to phosphorylation (Pho) of the MAP kinases p38 (A), ERK (B), and JNK (C). Rapamycin does not alter this pattern of MAP kinase activation. Total (Tot) levels of p38, ERK, and JNK remain unchanged. Blots are representative of at least 3 different experiments.

Circulation
Journal of the American Heart Association

Consistent with this observation, thrombin-induced mRNA levels were unchanged by rapamycin for up to 2 hours after stimulation. However, after 3 and 5 hours of thrombin stimulation, rapamycin increased mRNA levels compared with stimulation by thrombin alone. Taken together, these data imply that the enhancing effect of rapamycin on thrombin-induced TF expression initially occurs at the post-transcriptional level and hence, is independent of MAP kinase

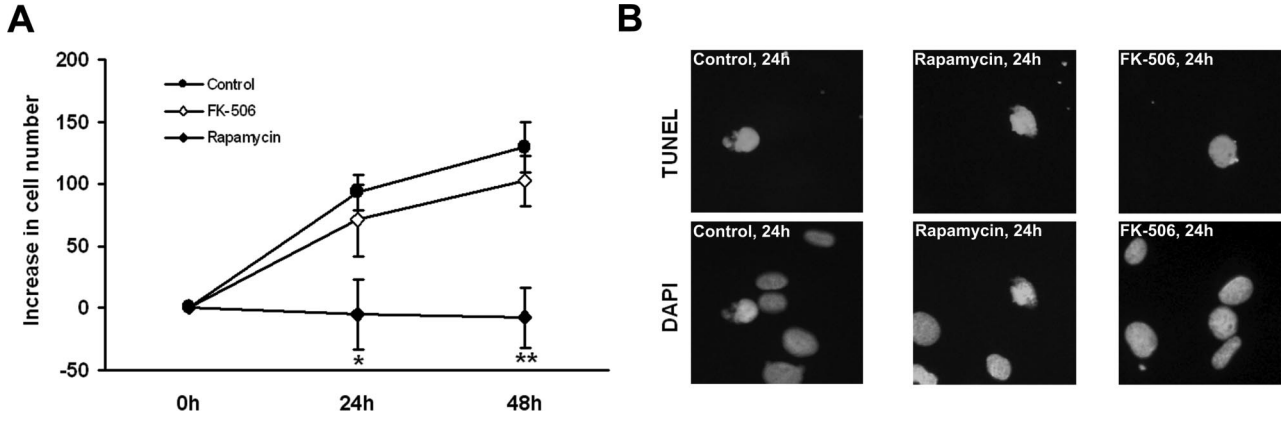


Figure 8. Rapamycin, but not FK-506, inhibits EC proliferation. A, Rapamycin completely inhibits EC proliferation induced by 10% FCS; in contrast, FK-506 does not significantly affect EC proliferation. *$P<0.05$ vs control; **$P<0.005$ vs control; and **$P<0.002$ vs FK-506. Three different experiments were performed in duplicate for each experimental condition. B, There was no increase in TUNEL-positive cells after incubation with rapamycin or FK-506 for 24 and 48 hours. Slides show representative TUNEL-positive cells with the corresponding DAPI staining after 24 hours of incubation with carrier (left), rapamycin (middle), and FK-506 (right).

activation, although a transcriptional effect of rapamycin cannot be ruled out at later time points. Indeed, mTOR is known to exert posttranscriptional effects, and TF expression can be regulated at both the transcriptional and posttranscriptional level.[47,48]

In summary, our study reveals that rapamycin, but not FK-506, enhances endothelial TF expression and reduces HAEC proliferation. These effects may favor the development of thrombus formation after deployment of sirolimus-eluting stents, particularly when antithrombotic drugs are withdrawn or ineffective, and may have interesting implications for the design of DESs.

Acknowledgments

This work was supported by the Swiss National Science Foundation (grant No. 3200B0-102232/1 to Dr Tanner and grant No. 3100-068118.02/1 to Dr Lüscher), the Bonizzi-Theler Foundation, the Hartmann-Müller Foundation, the Herzog-Egli Foundation, and the Olga Mayenfisch Foundation. The authors thank Dr F. Eberli and Dr W. Maier for discussion.

References

1. Schomig A, Kastrati A, Dirschinger J, Mehilli J, Schricke U, Pache J, Martinoff S, Neumann FJ, Schwaiger M. Coronary stenting plus platelet glycoprotein IIb/IIIa blockade compared with tissue plasminogen activator in acute myocardial infarction: Stent versus Thrombolysis for Occluded Coronary Arteries in Patients with Acute Myocardial Infarction Study Investigators. *N Engl J Med*. 2000;343:385–391.
2. Grines CL, Cox DA, Stone GW, Garcia E, Mattos LA, Giambartolomei A, Brodie BR, Madonna O, Eijgelshoven M, Lansky AJ, O'Neill WW, Morice MC. Coronary angioplasty with or without stent implantation for acute myocardial infarction: Stent Primary Angioplasty in Myocardial Infarction Study Group. *N Engl J Med*. 1999;341:1949–1956.
3. Lincoff AM, Califf RM, Moliterno DJ, Ellis SG, Ducas J, Kramer JH, Kleiman NS, Cohen EA, Booth JE, Sapp SK, Cabot CF, Topol EJ. Complementary clinical benefits of coronary-artery stenting and blockade of platelet glycoprotein IIb/IIIa receptors: Evaluation of Platelet IIb/IIIa Inhibition in Stenting Investigators. *N Engl J Med*. 1999;341:319–327.
4. Morice MC, Serruys PW, Sousa JE, Fajadet J, Ban Hayashi E, Perin M, Colombo A, Schuler G, Barragan P, Guagliumi G, Molnar F, Falotico R. A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. *N Engl J Med*. 2002;346:1773–1780.
5. Moses JW, Leon MB, Popma JJ, Fitzgerald PJ, Holmes DR, O'Shaughnessy C, Caputo RP, Kereiakes DJ, Williams DO, Teirstein PS, Jaeger JL, Kuntz RE. Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery. *N Engl J Med*. 2003;349:1315–1323.
6. Babapulle MN, Joseph L, Belisle P, Brophy JM, Eisenberg MJ. A hierarchical Bayesian meta-analysis of randomised clinical trials of drug-eluting stents. *Lancet*. 2004;364:583–591.
7. Gallo R, Padurean A, Jayaraman T, Marx S, Roque M, Adelman S, Chesebro J, Fallon J, Fuster V, Marks A, Badimon JJ. Inhibition of intimal thickening after balloon angioplasty in porcine coronary arteries by targeting regulators of the cell cycle. *Circulation*. 1999;99:2164–2170.
8. Grube E, Buellesfeld L. Rapamycin analogs for stent-based local drug delivery: everolimus- and tacrolimus-eluting stents. *Herz*. 2004;29:162–166.
9. Bartorelli AL, Trabattoni D, Fabbiocchi F, Montorsi P, de Martini S, Calligaris G, Teruzzi G, Galli S, Ravagnani P. Synergy of passive coating and targeted drug delivery: the tacrolimus-eluting Janus CarboStent. *J Interv Cardiol*. 2003;16:499–505.
10. McFadden EP, Stabile E, Regar E, Cheneau E, Ong AT, Kinnaird T, Suddath WO, Weissman NJ, Torguson R, Kent KM, Pichard AD, Satler LF, Waksman R, Serruys PW. Late thrombosis in drug-eluting coronary stents after discontinuation of antiplatelet therapy. *Lancet*. 2004;364:1519–1521.
11. Kereiakes DJ, Choo JK, Young JJ, Broderick TM. Thrombosis and drug-eluting stents: a critical appraisal. *Rev Cardiovasc Med*. 2004;5:9–15.
12. Eisenberg MJ. Drug-eluting stents: some bare facts. *Lancet*. 2004;364:1466–1467.
13. FDA advises physicians of adverse events associated with Cordis Cypher coronary stents. US Food and Drug Administration Public Health Web Notification. October 29, 2003:T03-T71.
14. Iakovou I, Schmidt T, Bonizzoni E, Ge L, Sangiorgi GM, Stankovic G, Airoldi F, Chieffo A, Montorfano M, Carlino M, Michev I, Corvaja N, Briguori C, Gerckens U, Grube E, Colombo A. Incidence, predictors, and outcome of thrombosis after successful implantation of drug-eluting stents. *JAMA*. 2005;293:2126–2130.
15. Honda Y, Fitzgerald PJ. Stent thrombosis: an issue revisited in a changing world. *Circulation*. 2003;108:2–5.
16. Moons AH, Levi M, Peters RJ. Tissue factor and coronary artery disease. *Cardiovasc Res*. 2002;53:313–325.
17. Sato Y, Asada Y, Marutsuka K, Hatakeyama K, Sumiyoshi A. Tissue factor induces migration of cultured aortic smooth muscle cells. *Thromb Haemost*. 1996;75:389–392.
18. Tutar E, Ozcan M, Kilickap M, Gulec S, Aras O, Pamir G, Oral D, Dandelet L, Key NS. Elevated whole-blood tissue factor procoagulant activity as a marker of restenosis after percutaneous transluminal coronary angioplasty and stent implantation. *Circulation*. 2003;108:1581–1584.
19. Marmur JD, Rossikhina M, Guha A, Fyfe B, Friedrich V, Mendlowitz M, Nemerson Y, Taubman MB. Tissue factor is rapidly induced in arterial smooth muscle after balloon injury. *J Clin Invest*. 1993;91:2253–2259.
20. Steffel J, Hermann M, Greutert H, Gay S, Luscher TF, Ruschitzka F, Tanner FC. Celecoxib decreases endothelial tissue factor expression through inhibition of c-Jun terminal NH_2 kinase phosphorylation. *Circulation*. 2005;111:1685–1689.
21. Tanner FC, Greutert H, Barandier C, Frischknecht K, Luscher TF. Different cell cycle regulation of vascular smooth muscle in genetic hypertension. *Hypertension*. 2003;42:184–188.
22. Steffel J, Akhmedov A, Greutert H, Lüscher TF, Tanner FC. Histamine induces tissue factor: implications for acute coronary syndromes. *Circulation*. 2005;112:341–349.
23. Tanner FC, Yang ZY, Duckers E, Gordon D, Nabel GJ, Nabel EG. Expression of cyclin-dependent kinase inhibitors in vascular disease. *Circ Res*. 1998;82:396–403.
24. Bogdanov VY, Balasubramanian V, Hathcock J, Vele O, Lieb M, Nemerson Y. Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. *Nat Med*. 2003;9:458–462.
25. Cui MZ, Zhao G, Winokur AL, Laag E, Bydash JR, Penn MS, Chisolm GM, Xu X. Lysophosphatidic acid induction of tissue factor expression in aortic smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2003;23:224–230.
26. Bavendiek U, Libby P, Kilbride M, Reynolds R, Mackman N, Schonbeck U. Induction of tissue factor expression in human endothelial cells by CD40 ligand is mediated via activator protein 1, nuclear factor-κB, and Egr-1. *J Biol Chem*. 2002;277:25032–25039.
27. Burnett PE, Barrow RK, Cohen NA, Snyder SH, Sabatini DM. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc Natl Acad Sci U S A*. 1998;95:1432–1437.
28. Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev*. 2004;18:1926–1945.
29. US Food and Drug Administration, Center for Devices and Radiological Health. Cypher Sirolimus-eluting coronary stent on RAPTOR over-the-wire delivery system or RAPTORRAIL rapid exchange delivery system. Available at http://www.fda.gov/cdrh/pdf2/p020026.html. Accessed December 17, 2004.
30. Gummert JF, Ikonen T, Morris RE. Newer immunosuppressive drugs: a review. *J Am Soc Nephrol*. 1999;10:1366–1380.
31. Klugherz BD, Llanos G, Lieuallen W, Kopia GA, Papandreou G, Narayan P, Sasseen B, Adelman SJ, Falotico R, Wilensky RL. Twenty-eight-day efficacy and pharmacokinetics of the sirolimus-eluting stent. *Coron Artery Dis*. 2002;13:183–188.
32. Suzuki T, Kopia G, Hayashi S, Bailey LR, Llanos G, Wilensky R, Klugherz BD, Papandreou G, Narayan P, Leon MB, Yeung AC, Tio F, Tsao PS, Falotico R, Carter AJ. Stent-based delivery of sirolimus reduces neointimal formation in a porcine coronary model. *Circulation*. 2001;104:1188–1193.
33. Asahara T, Chen D, Tsurumi Y, Kearney M, Rossow S, Passeri J, Symes JF, Isner JM. Accelerated restitution of endothelial integrity and endothelium-dependent function after phVEGF165 gene transfer. *Circulation*. 1996;94:3291–3302.

34. Tanaka H, Sukhova GK, Swanson SJ, Clinton SK, Ganz P, Cybulsky MI, Libby P. Sustained activation of vascular cells and leukocytes in the rabbit aorta after balloon injury. *Circulation*. 1993;88:1788–1803.
35. Schwartz RS, Chronos NA, Virmani R. Preclinical restenosis models and drug-eluting stents: still important, still much to learn. *J Am Coll Cardiol*. 2004;44:1373–1385.
36. Farb A, Sangiorgi G, Carter AJ, Walley VM, Edwards WD, Schwartz RS, Virmani R. Pathology of acute and chronic coronary stenting in humans. *Circulation*. 1999;99:44–52.
37. Grewe PH, Deneke T, Machraoui A, Barmeyer J, Muller KM. Acute and chronic tissue response to coronary stent implantation: pathologic findings in human specimen. *J Am Coll Cardiol*. 2000;35:157–163.
38. Matetzky S, Shenkman B, Guetta V, Shechter M, Bienart R, Goldenberg I, Novikov I, Pres H, Savion N, Varon D, Hod H. Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. *Circulation*. 2004;109: 3171–3175.
39. Leon C, Alex M, Klocke A, Morgenstern E, Moosbauer C, Eckly A, Spannagl M, Gachet C, Engelmann B. Platelet ADP receptors contribute to the initiation of intravascular coagulation. *Blood*. 2004;103:594–600.
40. Babinska A, Markell MS, Salifu MO, Akoad M, Ehrlich YH, Kornecki E. Enhancement of human platelet aggregation and secretion induced by rapamycin. *Nephrol Dial Transplant*. 1998;13:3153–3159.
41. Hofma SH, Van der Giessen WJ, Van Dalen B, Lemos PA, Serruys PW Prolonged endothelial dysfunction late after stenting with sirolimus-eluting stent compared to bare metal stent. *Eur Heart J*. 2004; 25(suppl):525. Abstract.
42. Eto M, Kozai T, Cosentino F, Joch H, Luscher TF. Statin prevents tissue factor expression in human endothelial cells: role of Rho/Rho-kinase and Akt pathways. *Circulation*. 2002;105:1756–1759.
43. Schecter AD, Spirn B, Rossikhina M, Giesen PL, Bogdanov V, Fallon JT, Fisher EA, Schnapp LM, Nemerson Y, Taubman MB. Release of active tissue factor by human arterial smooth muscle cells. *Circ Res*. 2000;87: 126–132.
44. Viswambharan H, Ming XF, Zhu S, Hubsch A, Lerch P, Vergeres G, Rusconi S, Yang Z. Reconstituted high-density lipoprotein inhibits thrombin-induced endothelial tissue factor expression through inhibition of RhoA and stimulation of phosphatidylinositol 3-kinase but not Akt/ endothelial nitric oxide synthase. *Circ Res*. 2004;94:918–925.
45. Bierer BE, Mattila PS, Standaert RF, Herzenberg LA, Burakoff SJ, Crabtree G, Schreiber SL. Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. *Proc Natl Acad Sci U S A*. 1990;87:9231–9235.
46. Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell*. 1991;66:807–815.
47. Deguchi H, Takeya H, Wada H, Gabazza EC, Hayashi N, Urano H, Suzuki K. Dilazep, an antiplatelet agent, inhibits tissue factor expression in endothelial cells and monocytes. *Blood*. 1997;90:2345–2356.
48. Lopez-Pedrera C, Jardi M, Ingles-Esteve J, Munoz-Canoves P, Dorado G, Velasco F, Felez J. Characterization of tissue factor expression on the human endothelial cell line ECV304. *Am J Hematol*. 1997;56:71–78.

American Heart Association.
Fighting Heart Disease and Stroke
Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Paclitaxel Enhances Thrombin-Induced Endothelial Tissue Factor Expression via c-Jun Terminal NH_2 Kinase Activation

Barbara E. Stähli,* Giovanni G. Camici,* Jan Steffel, Alexander Akhmedov, Kushiar Shoojati, Michelle Graber, Thomas F. Lüscher, Felix C. Tanner

Abstract—Paclitaxel is used on drug-eluting stents because it inhibits proliferation of vascular cells. Stent thrombosis remains a concern with this compound, particularly with higher dosages. This study investigates the effect of paclitaxel on tissue factor (TF) expression in human endothelial cells. Paclitaxel enhanced thrombin-induced endothelial TF protein expression in a concentration- and time-dependent manner. A concentration of 10^{-5} mol/L elicited a 2.1-fold increase in TF protein and a 1.6-fold increase in TF surface activity. The effect was similar after a 1 hour as compared with a 25-hour pretreatment period. Real-time polymerase chain reaction revealed that paclitaxel increased thrombin-induced TF mRNA expression. Paclitaxel potently activated c-Jun terminal NH_2 kinase (JNK) as compared with thrombin alone, whereas the thrombin-mediated phosphorylation of p38 and extracellular signal-regulated kinase remained unaffected. Similar to paclitaxel, docetaxel enhanced both TF expression and JNK activation as compared with thrombin alone. The JNK inhibitor SP600125 reduced thrombin-induced TF expression by 35%. Moreover, SP600125 blunted the effect of paclitaxel and docetaxel on thrombin-induced TF expression. Paclitaxel increases endothelial TF expression via its stabilizing effect on microtubules and selective activation of JNK. This observation provides novel insights into the pathogenesis of thrombus formation after paclitaxel-eluting stent deployment and may have an impact on drug-eluting stent design. (***Circ Res*. 2006;99:0-0.)**

Key Words: acute coronary syndrome ■ thrombosis ■ stents ■ MAP kinase ■ signal transduction

Percutaneous intervention is common practice for treating acute coronary syndromes.[1,2] Drug-eluting stents (DES), which are coated with antiproliferative agents, improve the outcome after coronary artery stenting.[3] Paclitaxel, a microtubule-stabilizing drug eliciting cell cycle arrest in G_2/M phase, is used on DES because it reduces vascular smooth muscle cell proliferation and migration.[4] Several randomized clinical trials have demonstrated that paclitaxel-eluting stents decrease intimal hyperplasia and restenosis, leading to reduced rates of major adverse cardiac events as compared with bare-metal stents (BMS).[3,5–7] In contrast, the use of DES has not reduced the occurrence of stent thrombosis as compared with BMS.[3,5,7–9] Acute, subacute, and late in-stent thromboses have been observed in patients treated with paclitaxel-eluting stents, particularly following cessation of clopidogrel therapy.[8,10–12] Moreover, the SCORE trial, which analyzed the effect of a stent releasing higher paclitaxel concentrations than the TAXUS stent, had to be terminated because of increased rates of in-stent thrombosis.[13] Although in-stent thrombosis after TAXUS stent implantation is less frequent, its rates may still be higher in "real world" patients than those reported in clinical trials.[7,10,11] In addition, if in-stent thrombosis occurs, it is associated with high morbidity and mortality.[14]

Tissue factor (TF) is a 47-kDa transmembrane glycoprotein-binding factor VIIa (FVIIa) and, in turn, activates FIX and FX[15,16]; thereby, the TF/FVIIa complex is the principal initiator of coagulation. It is well documented that TF is highly expressed in atherosclerotic plaques and that initiation of coagulation is a key event in the pathogenesis of acute coronary syndromes.[17] TF antigen and activity are indeed higher in plaques from patients with unstable angina or myocardial infarction than in those from patients with stable angina,[17,18] and increased TF plasma levels are found in unstable angina and acute myocardial infarction.[17,19,20] Given the important role of TF in acute coronary syndromes, it may be involved in the pathogenesis of in-stent thrombosis as well.

Rapamycin, another drug used with DES, does indeed enhance endothelial TF expression.[21] The effect of paclitaxel

Original received January 30, 2006; revision received April 25, 2006; accepted June 9, 2006.

From Cardiovascular Research (B.E.S., G.G.C., J.S., A.A., K.S., M.G., T.F.L., F.C.T.), Physiology Institute; and the Center for Integrative Human Physiology (B.E.S., G.G.C., J.S., A.A., K.S., M.G., T.F.L., F.C.T.), University of Zürich; and Cardiology (J.S., T.F.L., F.C.T.), Cardiovascular Center, University Hospital Zürich, Zürich, Switzerland.

*Both authors contributed equally to this study.

Correspondence to Felix C. Tanner, MD, Cardiovascular Research, Physiology Institute, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland. E-mail felix.tanner@access.unizh.ch

© 2006 American Heart Association, Inc.

Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/01.RES.0000233379.92010.fd**

on TF expression, however, is not known. This study was therefore designed to examine the effect of paclitaxel on TF expression in human endothelial cells.

Materials and Methods

Cell Culture and Morphology

Human aortic endothelial cells (HAECs) were purchased from Clonetics and cultured as described.[22] Cells were grown to confluence in 3-cm culture dishes, rendered quiescent for 24 hours in medium containing 0.5% FCS, and then stimulated with 1 U/mL thrombin (Sigma). Paclitaxel (Sigma and Alexis) was added to the dishes 1 hour before stimulation. To assess cytotoxicity, a colorimetric assay for detection of lactate dehydrogenase (LDH) was used according to the recommendations of the manufacturer (Roche). Cell morphology was evaluated by phase–contrast microscopy (Leitz DM IRB) at ×50 magnification and photographed (Olympus DP 50) without fixation.

Western Blot Analysis

Protein expression was determined by Western blot analysis as described.[23] Cells were lysed in 50 mmol/L Tris buffer, 25 μg was loaded per lane, and 10% SDS-PAGE was performed under reducing conditions. Resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) by semidry transfer. The antibody against human TF (American Diagnostica) was used at 1:2′000 dilution; antibodies against phosphorylated p38 mitogen-activated protein (MAP) kinase (p38), phosphorylated p44/42 MAP kinase (extracellular signal-regulated kinase [ERK]), and phosphorylated c-jun terminal NH_2 kinase (JNK) (all from Cell Signaling) were used at 1:1′000, 1:5′000, and 1:1′000 dilution, respectively. Antibodies against total p38, total ERK, and total JNK (all from Cell Signaling) were used at 1:2′000, 1:5′000, and 1:1′000 dilution, respectively. The antibody against IkB-α (Santa Cruz) was applied at 1:1′000 dilution. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used to ensure equal protein loading at an antibody (Chemicon) dilution of 1:20′000. Proteins were detected with a horseradish peroxidase–linked secondary antibody (Amersham).

Real-Time PCR

Total RNA was extracted with 1 mL TRIzol Reagent (Invitrogen) according to the recommendations of the manufacturer. Conversion of total cellular RNA to cDNA was performed with Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Amersham Bioscience) in a final volume of 33 μL using 4 μg of RNA. Real-time PCR amplification was performed in an MX3000P PCR cycler (Stratagene) using the SYBR Green JumpStart kit (Sigma) in 25 μL of final reaction volume containing 2 μL of cDNA, 10 pmol of each primer, 0.25 μL of internal reference dye, and 12.5 μL of JumpStart Taq ReadyMix (buffer, dNTP, stabilizers, SYBR Green, Taq polymerase, and JumpStart Taq antibody).[24] The total cDNA pool obtained served as template for subsequent PCR amplification with TF (F3)-specific primers (sense primer: 5′-TCCCCAGAGTTCACACCTTACC-3′; bases 508 to 529 of *F3* cDNA; National Center for Biotechnology Information [NCBI] no. NM 001993; antisense primer: 5′-TGACCACAAATACCACAGCTCC-3′; bases 892 to 913 of *F3* cDNA; NCBI no. NM 001993) using the following cycling parameters: 95° for 10 minutes for 1 cycle; 95° for 30 seconds, 60° for 1 minute, 72° for 1 minute, for a total of 40 cycles. A melting curve analysis was performed after amplification to verify the accuracy of the amplicon. L28 primers served as loading control. Products were separated by electrophoresis on a 1.6% agarose gel and visualized with ethidium bromide.

TF Surface Activity

TF surface activity was analyzed with a colorimetric assay (American Diagnostica) according to the recommendations of the manufacturer with some modifications as described.[25] Cells were grown to confluence in 12-well plates, stimulated with thrombin, washed twice with PBS, and incubated with human FVIIa and FX at 37°. This allowed the formation of a TF/FVIIa complex at the cell surface. This complex converted human FX to FXa, which was measured by its ability to cleave a chromogenic substrate. A standard curve was established with lipidated human TF to ensure that the results were in the linear range of detection (data not shown).

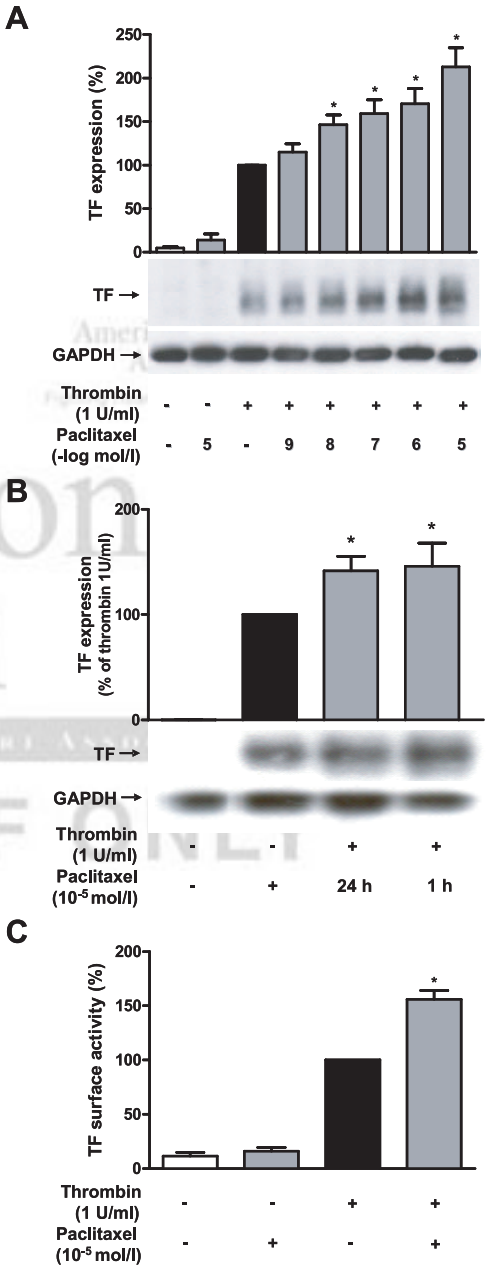


Figure 1. Paclitaxel enhances thrombin-induced TF protein expression. A, Paclitaxel enhances thrombin-induced TF protein expression in a concentration-dependent manner (n=6; *$P<0.0005$ vs thrombin alone). Values are given as percentage of TF expression in response to 5 hours of thrombin stimulation. Blots are normalized to GAPDH expression. B, Similar effect of paclitaxel on thrombin-induced TF protein expression after 1 hour and 25 hours of pretreatment (n=4; *$P<0.005$ vs thrombin alone, P=NS for 1 vs 25 hours). C, Paclitaxel enhances thrombin-induced TF surface activity (n=3; *$P<0.005$ vs thrombin alone). Values are given as percentage of TF surface activity in response to 5 hours of thrombin stimulation.

Circulation Research JOURNAL OF THE AMERICAN HEART ASSOCIATION FIRST PROOF ONLY

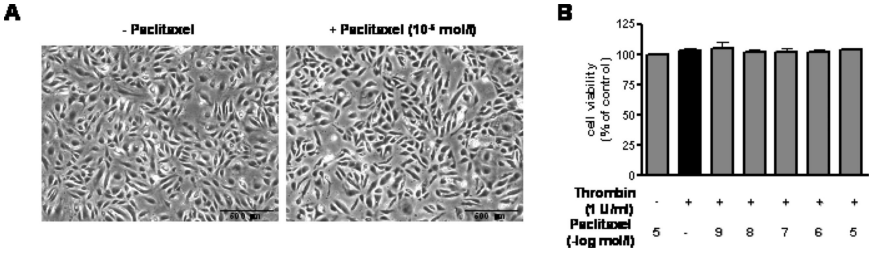


Figure 2. Lack of toxicity of paclitaxel. A, Morphology of HAECs after 5 hours of thrombin stimulation is similar without (left) and with (right) paclitaxel (10^{-5} mol/L). Magnification, ×50. B, LDH release reveals no cytotoxic effect of paclitaxel on HAECs at any concentration used (n≥5; *P*=NS).

Statistical Analysis

Data are reported as mean±SEM. Unpaired Student's *t* test was performed for statistical analysis. A probability value of <0.05 was considered to indicate a significant difference.

Results

Paclitaxel Enhances TF Protein Expression and Surface Activity

HAECs were stimulated with thrombin (1 U/mL) for 5 hours in the presence or absence of paclitaxel (10^{-9} to 10^{-5} mol/L). Thrombin induced a 20-fold increase in TF expression as compared with baseline (n=7; *P*<0.0001; Figure 1A). Pretreatment with paclitaxel enhanced thrombin-induced TF expression in a concentration-dependent manner; a maximal effect occurred at 10^{-5} mol/L and resulted in a 2.1-fold induction as compared with thrombin alone, corresponding to a 43.3-fold induction as compared with baseline (n=6; *P*<0.0005; Figure 1A). The effect of paclitaxel was similar after a 1 hour as compared with a 25 hour pretreatment period (n=4; *P*=NS for 1 versus 25 hours; Figure 1B). The effect of paclitaxel was first observed after 3 hours and elicited a significant increase in thrombin-induced TF expression after 5 and 7 hours (n=4; *P*<0.05; Figure 3B). In another time-course analysis, thrombin-induced TF expression was maximal after 6 hours of stimulation and decreased to 50% after 12 hours, 32% after 18 hours, and 27% after 24 hours; paclitaxel significantly enhanced thrombin-induced TF expression by 2.2-fold after 6 hours (n=4; *P*<0.05) and by 1.4-fold after 12 hours (n=4; *P*<0.05), although its effect did not reach statistical significance after 18 and 24 hours (n=4; *P*=NS) (data not shown). Consistent with these observations, paclitaxel enhanced thrombin-induced TF surface activity by 56% (n=3; *P*<0.005; Figure 1C). Paclitaxel alone did not affect basal TF expression (n=7; *P*=NS; Figure 1A). Paclitaxel did not affect endothelial cell morphology (Figure 2A) nor LDH release (Figure 2B) at any concentration used (n≥5; *P*=NS).

Paclitaxel Enhances TF mRNA Expression

Real-time PCR revealed that thrombin (1 U/mL) induced TF mRNA expression with a maximal effect occurring after 2 hours. Pretreatment with paclitaxel (10^{-5} mol/L) enhanced thrombin-induced TF mRNA expression by 1.6-fold after 1 hour (n≥5; *P*<0.05; Figure 3A), by 1.5-fold after 2 hours (n≥5; *P*=0.09; Figure 3A), and by 1.7-fold after 3 hours (n≥5; *P*=0.21; Figure 3A).

Paclitaxel Selectively Activates JNK

Thrombin induced a transient phosphorylation of the MAP kinases p38, ERK, and JNK. Maximal activation of JNK was observed after 60 minutes, whereas that of p38 and ERK occurred after 5 minutes.[21] Paclitaxel significantly increased JNK phosphorylation after 15, 30, and 60 minutes by 3.7-, 3.2-, and 2.0-fold, respectively, as compared with thrombin alone (n=4; *P*<0.05 for each time point; Figure 4A). Phosphorylation of p38 was slightly prolonged by paclitaxel after 15 minutes of stimulation (n=4; *P*<0.005; Figure 4B), whereas all of the other time points remained unaltered (n=4; *P*=NS; Figure 4B). Phosphorylation of ERK was not affected by paclitaxel, except for a slight decrease at the 5-minute time point (n=4; *P*<0.01; Figure 4C). Neither thrombin nor paclitaxel altered total expression of MAP kinases. Thrombin-induced IkB-α degradation was not affected by pretreatment with paclitaxel (n=3; *P*=NS; data not shown).

Paclitaxel and Docetaxel Exert Similar Effects on Both TF and JNK

HAECs were stimulated with thrombin (1 U/mL) for 5 hours in the presence or absence of paclitaxel or docetaxel (both at 10^{-6} and 10^{-5} mol/L). Similar to paclitaxel, docetaxel enhanced thrombin-induced TF expression by 2.2-fold as compared with thrombin alone (n≥4; *P*<0.05 for thrombin plus paclitaxel versus thrombin alone; *P*<0.005 for thrombin plus docetaxel versus thrombin alone; *P*=NS for thrombin plus paclitaxel versus thrombin plus docetaxel; Figure 5A). Docetaxel did not affect endothelial cell morphology nor LDH release at any concentration used (n=3; *P*=NS; data not shown).

Similar to paclitaxel, docetaxel enhanced JNK phosphorylation as compared with thrombin alone. The increase in JNK activation after 2 hours of thrombin stimulation was 3.2-fold for paclitaxel and 2.2-fold for docetaxel (n≥4; *P*=0.0001 for thrombin plus paclitaxel versus thrombin alone; *P*<0.05 for thrombin plus docetaxel versus thrombin alone; *P*=NS for thrombin plus paclitaxel versus thrombin plus docetaxel; Figure 5B).

JNK Mediates the Effect of Paclitaxel and Docetaxel on TF

HAECs were pretreated with SP600125, a specific inhibitor of JNK, 90 minutes before stimulation with thrombin (1 U/mL). SP600125 (10^{-6} mol/L) reduced thrombin-induced TF expression by 35% (n=3; *P*<0.01; Figure 5C). Moreover, SP600125 reduced the effect of paclitaxel on thrombin-induced TF expression by 110% and that of docetaxel by 105%, respectively (n=4; *P*<0.0001 for paclitaxel versus paclitaxel plus SP600125; *P*<0.05 for docetaxel versus docetaxel plus SP600125; Figure 5D). Hence, inhibition of JNK by SP600125 blunted the effect of paclitaxel and docetaxel on thrombin-induced TF expression (n=4; *P*=NS

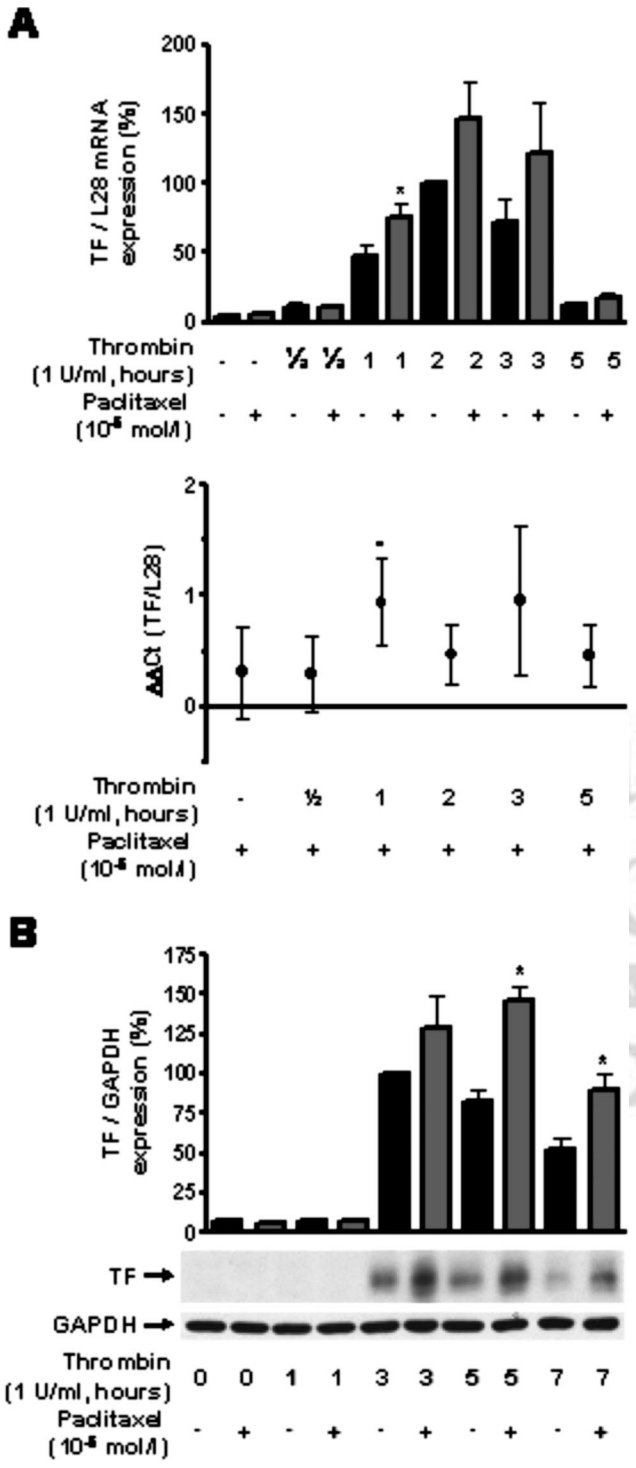


Figure 3. Paclitaxel enhances thrombin-induced TF mRNA expression. A, top, Paclitaxel enhances thrombin-induced TF mRNA expression in a time-dependent manner (n≥5; $P<0.05$ vs thrombin alone). TF mRNA levels are normalized to L28; values are indicated as percent of TF mRNA expression induced by 2 hours of thrombin stimulation. Bottom, ΔΔCt values assessing the effect of paclitaxel on thrombin-induced TF mRNA expression at each time point (n≥5; *$P<0.05$ vs thrombin alone). B, Effect of paclitaxel on thrombin-induced TF protein expression is time dependent. A significant increase is observed after incubation with paclitaxel for 5 and 7 hours (n=4; *$P<0.05$ vs thrombin alone). Values are given as percent of TF expression in response to 3 hours of thrombin stimulation. Blots are normalized to GAPDH expression.

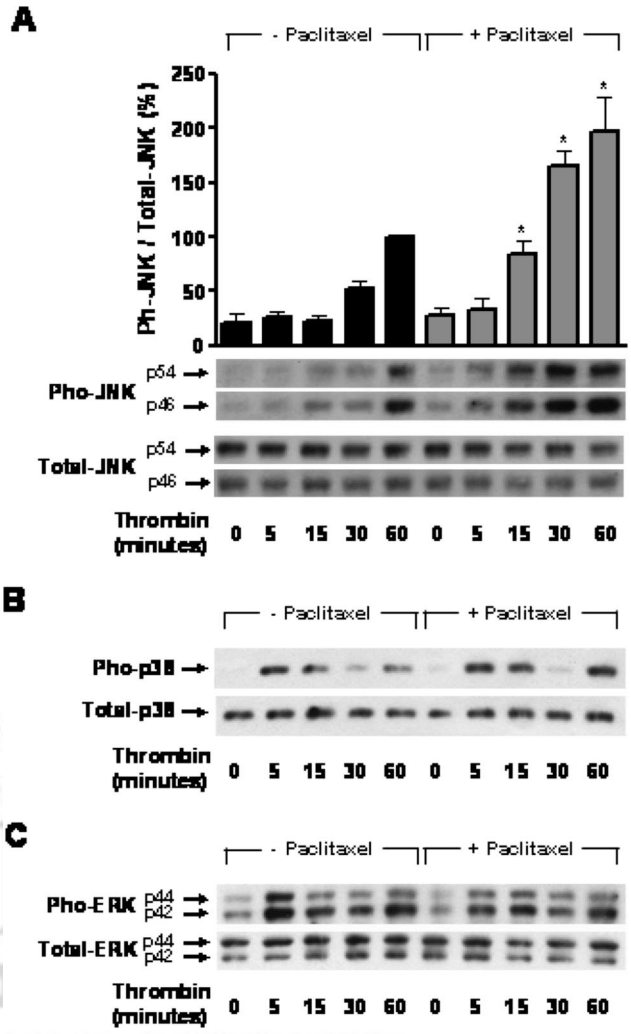


Figure 4. Paclitaxel enhances thrombin-induced JNK activation. A, Paclitaxel (10^{-5} mol/L) enhances thrombin-induced JNK activation after 15, 30, and 60 minutes of stimulation (n=4; *$P<0.05$ vs thrombin alone). Expression of total JNK is not affected. B, Paclitaxel (10^{-5} mol/L) slightly prolongs maximal p38 phosphorylation. Expression of total p38 is not affected. C, Paclitaxel (10^{-5} mol/L) slightly decreases maximal ERK phosphorylation. Expression of total ERK is not affected.

for paclitaxel plus SP600125 versus thrombin alone; P=NS for docetaxel plus SP600125 versus thrombin alone; Figure 5D).

Discussion

This study reveals that paclitaxel enhances thrombin-induced endothelial TF protein expression and surface activity in a concentration- as well as time-dependent manner via its stabilizing effect on microtubules and selective activation of JNK.

Paclitaxel is a lipophilic diterpenoid that binds to the β subunit of the tubulin heterodimer; this interaction promotes tubulin polymerization leading to the formation of stable nonfunctional microtubule bundles and promoting cell cycle arrest in G_2/M phase.[26,27] Via this mechanism, paclitaxel inhibits proliferation as well as migration of vascular smooth muscle cells and reduces restenosis rates in patients with

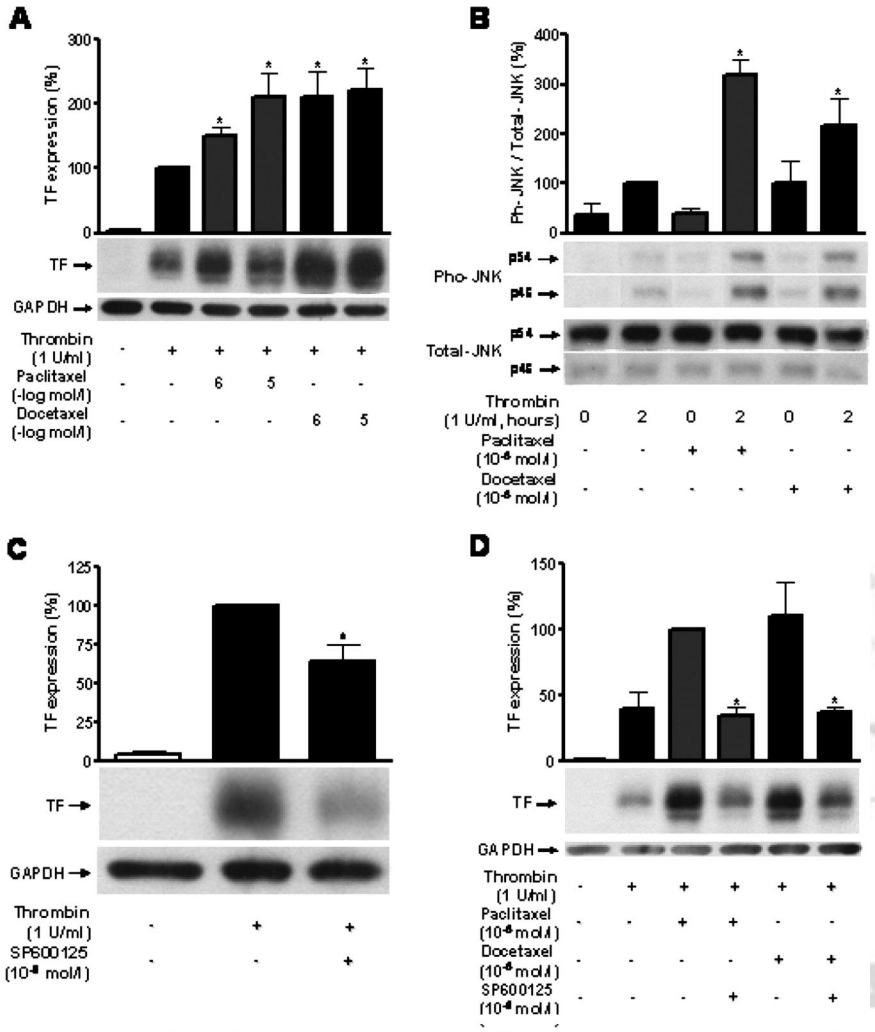


Figure 5. JNK mediates the effect of paclitaxel and docetaxel on TF expression. A, Similar to paclitaxel, docetaxel (10^{-6} and 10^{-5} mol/L) enhances thrombin-induced TF expression (n≥4; *$P<0.05$ for thrombin+paclitaxel vs thrombin alone; *$P<0.005$ for thrombin+docetaxel vs thrombin alone; P=NS for thrombin+paclitaxel vs thrombin+docetaxel). Values are given as percentage of TF expression in response to 5 hours of thrombin stimulation. Blots are normalized to GAPDH expression. B, Paclitaxel and docetaxel enhance JNK phosphorylation as compared with thrombin alone (n≥4; *P=0.0001 for thrombin+paclitaxel vs thrombin alone; *$P<0.05$ for thrombin+docetaxel vs thrombin alone; P=NS for thrombin+docetaxel vs thrombin+paclitaxel). Expression of total JNK is not affected. C, SP600125 (10^{-6} mol/L) reduces thrombin-induced TF expression to 65% of control (n=3; $P<0.01$). D, In the presence of SP600125 (10^{-6} mol/L), the effect of paclitaxel and docetaxel on thrombin-induced TF expression is blunted (n=4; $P<0.0001$ for paclitaxel vs paclitaxel+SP600125; $P<0.05$ for docetaxel vs docetaxel+SP600125; P=NS for paclitaxel+SP600125 vs thrombin alone; P=NS for docetaxel+SP600125 vs thrombin alone).

FIRST PROOF ONLY

coronary artery disease.[3,4,7] Because of its lipophilic properties, paclitaxel accumulates in the vessel wall, reaching particularly high concentrations in the intima[28,29]; local tissue concentrations are indeed 100-fold higher as compared with perfusate concentrations during ex vivo endovascular perfusion.[28] In a porcine coronary artery stent model, tissue concentrations of paclitaxel reached 3.2 μg/g arterial tissue after 28 days and drop below detection limit within 3 months only[30]; this tissue concentration of paclitaxel corresponds to 3.7×10^{-6} mol/L at an assumed tissue density of 1 g/cm^3.[30] Similar tissue concentrations have been measured in a rabbit iliac artery stent model.[31] Thus, the paclitaxel concentrations used in our study are comparable to local tissue concentrations after stent deployment.

In animal models, partial reendothelialization has been observed as early as 4 days after DES deployment, whereas complete reendothelialization occurs within 3 weeks.[30,32] In humans, partial reendothelialization has been documented 2 weeks after stenting and is usually completed within 12 weeks.[32,33] Paclitaxel-eluting stents have a biphasic drug-release profile in vitro, characterized by an initial burst during the first 48 hours after implantation, followed by a sustained low-level release for at least 2 weeks.[6,34,35] Because of its lipophilic properties, however, very high paclitaxel concentrations have been measured up to 4 weeks after stent implantation in vivo, and the drug remains detectable for up to 12 weeks. Therefore, the time course of reendothelialization coincides with the presence of paclitaxel in the vessel wall after stent deployment. Thus, paclitaxel may indeed alter the biology of the endothelium within the stented area.

In-stent thrombosis has been described in patients treated with paclitaxel-eluting stents, particularly after cessation of antiplatelet therapy.[8,10,12] Our data demonstrate that the effect of paclitaxel is maintained over prolonged time periods and that it becomes effective as soon as a stimulus like thrombin is present; hence, the data are consistent with the clinical observation that cessation of antiplatelet therapy is a risk factor for thrombosis of drug-eluting stents. Moreover, in view of the coronary paclitaxel concentrations after stent deployment, as well as the time course of reendothelialization, paclitaxel may indeed contribute to the development of subacute or late in-stent thrombosis by enhancing endothelial TF expression. This interpretation is supported by the results of the SCORE trial, which compared the QuaDDS stent (coated with the paclitaxel-derivative 7-hexanoyltaxol) to BMS; the trial had to be terminated prematurely because of very high rates of subacute and late in-stent thrombosis as

well as major adverse cardiac events.[13] The increased rates of in-stent thrombosis have been primarily related to the high paclitaxel doses released by these stents, although an unfavorable effect of the stent design may have contributed.[13] Interestingly, the paclitaxel derivative 7-hexanoyltaxol can be detected up to 10 mm proximal and distal to the stent margins, suggesting that paclitaxel may induce TF expression in the vessel segments proximal and distal to the stent as well.[36]

The effect of paclitaxel was attributable to a specific action on endothelial cell function, as it neither affected cell morphology nor induced any toxicity.[37,38] This is consistent with previous observations demonstrating that paclitaxel (10^{-5} mol/L) does not induce any cell death in human pulmonary artery endothelial cells or aortic smooth muscle cells after 16 and 36 hours of incubation, respectively.[38,39] The increase in TF surface activity was less pronounced than that in protein expression, which may be related to the presence of inactive encrypted TF on the cell surface or to the distribution of TF in several cellular compartments.[40]

Thrombin induces TF expression at the transcriptional level via activation of the MAP kinases p38, ERK, and JNK.[21,41] The increase in TF protein expression by paclitaxel was preceded by an enhanced TF mRNA expression. Consistent with this observation, paclitaxel augmented thrombin-induced JNK phosphorylation. Interestingly, the activation pattern of p38 and ERK was not affected, indicating that paclitaxel selectively activates JNK without affecting other signal-transduction molecules in endothelial cells. Consistent with this interpretation, IkB-α degradation was not altered by paclitaxel. Similar observations have been made in different cancer cell lines, demonstrating that the effect of paclitaxel on JNK activation is not restricted to the endothelium.[42–44] To assess whether JNK indeed mediates the induction of TF expression in response to thrombin and, in particular, to paclitaxel, endothelial cells were pretreated with SP600125, a selective inhibitor of JNK catalytic activity. SP600125 decreased thrombin-induced TF expression by approximately a third, indicating that JNK is not the only signal-transduction mediator regulating thrombin-induced TF expression. In contrast, the JNK inhibitor fully prevented the effect of paclitaxel, strongly suggesting that the effect of paclitaxel on TF expression is selectively mediated by the JNK pathway. However, it cannot be ruled out completely that other signal-transduction pathways may be involved as well.

The microtubule-stabilizing agent docetaxel was used to elucidate whether the increase in thrombin-induced JNK activation and TF expression by paclitaxel was related to perturbation of microtubule function.[43] Both microtubule-stabilizing agents exerted a similar effect on JNK activation and TF expression, and SP600125 blunted the enhancing effect of both docetaxel and paclitaxel on thrombin-induced TF expression. Thus, the action of paclitaxel on JNK activation and TF expression seems to depend on stabilization of microtubule bundles rather than on a substance-specific effect. Consistent with this interpretation, JNK activation in response to changes in the microtubule cytoskeleton has been described in different cancer cell lines.[43,44]

Paclitaxel enhanced thrombin-induced endothelial TF expression by 2.1-fold. Rapamycin augmented thrombin-induced TF expression to a similar extent, whereas the mechanisms of action of the 2 drugs differ completely: binding of rapamycin to its intracellular receptor FKBP-12 abrogates p70S6 kinase phosphorylation, leading to enhanced endothelial TF expression; whereas JNK activation remains unaffected.[21] Large-scale clinical trials have demonstrated that patients receiving paclitaxel-eluting stents have similar rates of in-stent thrombosis as compared with rapamycin-eluting stents.[11,45] This observation is consistent with the similar degree of TF induction in endothelial cells, suggesting that the latter may indeed be importantly involved in thrombosis of DES.

In conclusion, this study indicates that paclitaxel increases endothelial TF expression via JNK activation because of its microtubule stabilizing effect. The enhanced endothelial TF expression may favor thrombus formation after paclitaxel-eluting stent deployment, particularly when antithrombotic drugs are withdrawn or thrombin levels are elevated as it occurs in acute coronary syndromes. Therefore, these findings may have interesting implications for drug-eluting stent design.

Sources of Funding

This work was supported by the Swiss National Science Foundation (grant No. 3200B0-102232/1 to F.C.T. and grant No. 3100-068118.02/1 to T.F.L.), the Bonizzi-Theler Foundation, the Hartmann-Müller Foundation, the Olga Mayenfisch Foundation, the Herzog-Egli Foundation, and the Swiss Heart Foundation.

Disclosures

None.

References

1. Lincoff AM, Califf RM, Moliterno DJ, Ellis SG, Ducas J, Kramer JH, Kleiman NS, Cohen EA, Booth JE, Sapp SK, Cabot CF, Topol EJ. Complementary clinical benefits of coronary-artery stenting and blockade of platelet glycoprotein IIb/IIIa receptors. Evaluation of Platelet IIb/IIIa Inhibition in Stenting Investigators. *N Engl J Med*. 1999;341:319–327.
2. Grines CL, Cox DA, Stone GW, Garcia E, Mattos LA, Giambartolomei A, Brodie BR, Madonna O, Eijgelshoven M, Lansky AJ, O'Neill WW, Morice MC. Coronary angioplasty with or without stent implantation for acute myocardial infarction. Stent Primary Angioplasty in Myocardial Infarction Study Group. *N Engl J Med*. 1999;341:1949–1956.
3. Babapulle MN, Joseph L, Belisle P, Brophy JM, Eisenberg MJ. A hierarchical Bayesian meta-analysis of randomised clinical trials of drug-eluting stents. *Lancet*. 2004;364:583–591.
4. Sollott SJ, Cheng L, Pauly RR, Jenkins GM, Monticone RE, Kuzuya M, Froehlich JP, Crow MT, Lakatta EG, Rowinsky EK, Kinsella JL. Taxol inhibits neointimal smooth muscle cell accumulation after angioplasty in the rat. *J Clin Invest*. 1995;95:1869–1876.
5. Stone GW, Ellis SG, Cox DA, Hermiller J, O'Shaughnessy C, Mann JT, Turco M, Caputo R, Bergin P, Greenberg J, Popma JJ, Russell ME. One-year clinical results with the slow-release, polymer-based, paclitaxel-eluting TAXUS stent: the TAXUS-IV trial. *Circulation*. 2004; 109:1942–1947.
6. Colombo A, Drzewiecki J, Banning A, Grube E, Hauptmann K, Silber S, Dudek D, Fort S, Schiele F, Zmudka K, Guagliumi G, Russell ME. Randomized study to assess the effectiveness of slow- and moderate-release polymer-based paclitaxel-eluting stents for coronary artery lesions. *Circulation*. 2003;108:788–794.
7. Stone GW, Ellis SG, Cox DA, Hermiller J, O'Shaughnessy C, Mann JT, Turco M, Caputo R, Bergin P, Greenberg J, Popma JJ, Russell ME. A polymer-based, paclitaxel-eluting stent in patients with coronary artery disease. *N Engl J Med*. 2004;350:221–231.

8. McFadden EP, Stabile E, Regar E, Cheneau E, Ong AT, Kinnaird T, Suddath WO, Weissman NJ, Torguson R, Kent KM, Pichard AD, Satler LF, Waksman R, Serruys PW. Late thrombosis in drug-eluting coronary stents after discontinuation of antiplatelet therapy. *Lancet*. 2004;364: 1519–1521.
9. Eisenberg MJ. Drug-eluting stents: some bare facts. *Lancet*. 2004;364: 1466–1467.
10. Iakovou I, Schmidt T, Bonizzoni E, Ge L, Sangiorgi GM, Stankovic G, Airoldi F, Chieffo A, Montorfano M, Carlino M, Michev I, Corvaja N, Briguori C, Gerckens U, Grube E, Colombo A. Incidence, predictors, and outcome of thrombosis after successful implantation of drug-eluting stents. *JAMA*. 2005;293:2126–2130.
11. Windecker S, Remondino A, Eberli FR, Juni P, Raber L, Wenaweser P, Togni M, Billinger M, Tuller D, Seiler C, Roffi M, Corti R, Sutsch G, Maier W, Luscher T, Hess OM, Egger M, Meier B. Sirolimus-eluting and paclitaxel-eluting stents for coronary revascularization. *N Engl J Med*. 2005;353:653–662.
12. Ong AT, McFadden EP, Regar E, de Jaegere PP, van Domburg RT, Serruys PW. Late angiographic stent thrombosis (LAST) events with drug-eluting stents. *J Am Coll Cardiol*. 2005;45:2088–2092.
13. Grube E, Lansky A, Hauptmann KE, Di Mario C, Di Sciascio G, Colombo A, Silber S, Stumpf J, Reifart N, Fajadet J, Marzocchi A, Schofer J, Dumas P, Hoffmann R, Guagliumi G, Pitney M, Russell ME. High-dose 7-hexanoyltaxol-eluting stent with polymer sleeves for coronary revascularization: one-year results from the SCORE randomized trial. *J Am Coll Cardiol*. 2004;44:1368–1372.
14. Ong AT, Hoye A, Aoki J, van Mieghem CA, Rodriguez Granillo GA, Sonnenschein K, Regar E, McFadden EP, Sianos G, van der Giessen WJ, de Jaegere PP, de Feyter P, van Domburg RT, Serruys PW. Thirty-day incidence and six-month clinical outcome of thrombotic stent occlusion after bare-metal, sirolimus, or paclitaxel stent implantation. *J Am Coll Cardiol*. 2005;45:947–953.
15. Tremoli E, Camera M, Toschi V, Colli S. Tissue factor in atherosclerosis. *Atherosclerosis*. 1999;144:273–283.
16. Mackman N. Role of tissue factor in hemostasis, thrombosis, and vascular development. *Arterioscler Thromb Vasc Biol*. 2004;24:1015–1022.
17. Moons AH, Levi M, Peters RJ. Tissue factor and coronary artery disease. *Cardiovasc Res*. 2002;53:313–325.
18. Ardissino D, Merlini PA, Ariens R, Coppola R, Bramucci E, Mannucci PM. Tissue-factor antigen and activity in human coronary atherosclerotic plaques. *Lancet*. 1997;349:769–771.
19. Misumi K, Ogawa H, Yasue H, Soejima H, Suefuji H, Nishiyama K, Takazoe K, Kugiyama K, Tsuji I, Kumeda K, Nakamura S. Comparison of plasma tissue factor levels in unstable and stable angina pectoris. *Am J Cardiol*. 1998;81:22–26.
20. Suefuji H, Ogawa H, Yasue H, Kaikita K, Soejima H, Motoyama T, Mizuno Y, Oshima S, Saito T, Tsuji I, Kumeda K, Kamikubo Y, Nakamura S. Increased plasma tissue factor levels in acute myocardial infarction. *Am Heart J*. 1997;134:253–259.
21. Steffel J, Latini RA, Akhmedov A, Zimmermann D, Zimmerling P, Luscher TF, Tanner FC. Rapamycin, but not FK-506, increases endothelial tissue factor expression: implications for drug-eluting stent design. *Circulation*. 2005;112:2002–2011.
22. Steffel J, Akhmedov A, Greutert H, Luscher TF, Tanner FC. Histamine induces tissue factor expression: implications for acute coronary syndromes. *Circulation*. 2005;112:341–349.
23. Steffel J, Hermann M, Greutert H, Gay S, Luscher TF, Ruschitzka F, Tanner FC. Celecoxib decreases endothelial tissue factor expression through inhibition of c-Jun terminal NH2 kinase phosphorylation. *Circulation*. 2005;111:1685–1689.
24. Bogdanov VY, Balasubramanian V, Hathcock J, Vele O, Lieb M, Nemerson Y. Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. *Nat Med*. 2003;9:458–462.
25. Cui MZ, Zhao G, Winokur AL, Laag E, Bydash JR, Penn MS, Chisolm GM, Xu X. Lysophosphatidic acid induction of tissue factor expression in aortic smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2003;23: 224–230.
26. Rowinsky EK, Donehower RC. Paclitaxel (taxol). *N Engl J Med*. 1995; 332:1004–1014.
27. Crown J, O'Leary M. The taxanes: an update. *Lancet*. 2000;355: 1176–1178.
28. Creel CJ, Lovich MA, Edelman ER. Arterial paclitaxel distribution and deposition. *Circ Res*. 2000;86:879–884.
29. Levin AD, Vukmirovic N, Hwang CW, Edelman ER. Specific binding to intracellular proteins determines arterial transport properties for rapamycin and paclitaxel. *Proc Natl Acad Sci U S A*. 2004;101: 9463–9467.
30. Vogt F, Stein A, Rettemeier G, Krott N, Hoffmann R, vom Dahl J, Bosserhoff AK, Michaeli W, Hanrath P, Weber C, Blindt R. Long-term assessment of a novel biodegradable paclitaxel-eluting coronary polylactide stent. *Eur Heart J*. 2004;25:1330–1340.
31. Finn AV, Kolodgie FD, Harnek J, Guerrero LJ, Acampado E, Tefera K, Skorija K, Weber DK, Gold HK, Virmani R. Differential response of delayed healing and persistent inflammation at sites of overlapping sirolimus- or paclitaxel-eluting stents. *Circulation*. 2005;112:270–278.
32. Schwartz RS, Chronos NA, Virmani R. Preclinical restenosis models and drug-eluting stents: still important, still much to learn. *J Am Coll Cardiol*. 2004;44:1373–1385.
33. Grewe PH, Deneke T, Machraoui A, Barmeyer J, Muller KM. Acute and chronic tissue response to coronary stent implantation: pathologic findings in human specimen. *J Am Coll Cardiol*. 2000;35:157–163.
34. Halkin A, Stone GW. Polymer-based paclitaxel-eluting stents in percutaneous coronary intervention: a review of the TAXUS trials. *J Interv Cardiol*. 2004;17:271–282.
35. Ranade SV, Miller KM, Richard RE, Chan AK, Allen MJ, Helmus MN. Physical characterization of controlled release of paclitaxel from the TAXUS Express2 drug-eluting stent. *J Biomed Mater Res A*. 2004;71: 625–634.
36. de la Fuente LM, Miano J, Mrad J, Penaloza E, Yeung AC, Eury R, Froix M, Fitzgerald PJ, Stertzer SH. Initial results of the Quanam drug eluting stent (QuaDS-QP-2) Registry (BARDDS) in human subjects. *Catheter Cardiovasc Interv*. 2001;53:480–488.
37. Coomber BL, Gotlieb AI. In vitro endothelial wound repair. Interaction of cell migration and proliferation. *Arteriosclerosis*. 1990;10:215–222.
38. Blagosklonny MV, Darzynkiewicz Z, Halicka HD, Pozarowski P, Demidenko ZN, Barry JJ, Kamath KR, Herrmann RA. Paclitaxel induces primary and postmitotic G1 arrest in human arterial smooth muscle cells. *Cell Cycle*. 2004;3:1050–1056.
39. Petrache I, Birukova A, Ramirez SI, Garcia JG, Verin AD. The role of the microtubules in tumor necrosis factor-alpha-induced endothelial cell permeability. *Am J Respir Cell Mol Biol*. 2003;28:574–581.
40. Schecter AD, Giesen PL, Taby O, Rosenfield CL, Rossikhina M, Fyfe BS, Kohtz DS, Fallon JT, Nemerson Y, Taubman MB. Tissue factor expression in human arterial smooth muscle cells. TF is present in three cellular pools after growth factor stimulation. *J Clin Invest*. 1997;100: 2276–2285.
41. Liu Y, Pelekanakis K, Woolkalis MJ. Thrombin and tumor necrosis factor alpha synergistically stimulate tissue factor expression in human endothelial cells: regulation through c-Fos and c-Jun. *J Biol Chem*. 2004;279: 36142–36147.
42. Lee LF, Li G, Templeton DJ, Ting JP. Paclitaxel (Taxol)-induced gene expression and cell death are both mediated by the activation of c-Jun NH2-terminal kinase (JNK/SAPK). *J Biol Chem*. 1998;273:28253–28260.
43. Wang TH, Wang HS, Ichijo H, Giannakakou P, Foster JS, Fojo T, Wimalasena J. Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. *J Biol Chem*. 1998;273:4928–4936.
44. Yujiri T, Fanger GR, Garrington TP, Schlesinger TK, Gibson S, Johnson GL. MEK kinase 1 (MEKK1) transduces c-Jun NH2-terminal kinase activation in response to changes in the microtubule cytoskeleton. *J Biol Chem*. 1999;274:12605–12610.
45. Kastrati A, Dibra A, Eberle S, Mehilli J, Suarez de Lezo J, Goy JJ, Ulm K, Schomig A. Sirolimus-eluting stents vs paclitaxel-eluting stents in patients with coronary artery disease: meta-analysis of randomized trials. *JAMA*. 2005;294:819–825.

Tissue Factor in Cardiovascular Diseases
Molecular Mechanisms and Clinical Implications

Jan Steffel, MD; Thomas F. Lüscher, MD; Felix C. Tanner, MD

Abstract—Tissue factor (TF), formerly known as thromboplastin, is the key initiator of the coagulation cascade; it binds factor VIIa resulting in activation of factor IX and factor X, ultimately leading to fibrin formation. TF expression and activity can be induced in endothelial cells, vascular smooth muscle cells, and monocytes by various stimuli such as cytokines, growth factors, and biogenic amines. These mediators act through diverse signal transduction mechanisms including MAP kinases, PI3-kinase, and protein kinase C. Cellular TF is present in three pools as surface, encrypted, and intracellular protein. TF can also be detected in the bloodstream, referred to as circulating or blood-borne TF. Elevated levels of TF are observed in patients with cardiovascular risk factors such as hypertension, diabetes, dyslipidemia, and smoking as well as in those with acute coronary syndromes. TF may indeed be involved in the pathogenesis of atherosclerosis by promoting thrombus formation; in addition, it can induce migration and proliferation of vascular smooth muscle cells. As a consequence, therapeutic strategies have been developed to specifically interfere with the action of TF such as antibodies against TF, site-inactivated factor VIIa, or recombinant TF pathway inhibitor. Inhibition of TF action appears to be an attractive target for the treatment of cardiovascular diseases. (***Circulation*****. 2006; 113:722-731.)**

Key Words: atherosclerosis ■ coagulation ■ myocardial infarction ■ thrombosis

Tissue factor (TF) has long been known as a key initiator of the coagulation cascade. Over the last decade, our understanding of the molecular regulation of TF expression in vascular cells has profoundly improved; moreover, TF has been recognized to be involved in the pathogenesis of cardiovascular diseases. Therefore, therapeutic strategies are being developed to specifically interfere with TF and its effectors.

We will review the molecular regulation of TF expression emphasizing the role of TF in cardiovascular diseases as well as the resulting implications for the treatment of atherosclerosis and acute coronary syndromes.

Molecular Mechanisms of Tissue Factor Expression

Tissue factor, formerly known as thromboplastin, is a 47-kDa protein expressed in both vascular and nonvascular cells. The TF gene is located on chromosome 1 and consists of 6 exons.[1] One main transcript as well as at least one alternatively spliced form have been described.[2] In the vessel wall, TF is constitutively expressed in subendothelial cells such as vascular smooth muscle cells leading to rapid initiation of coagulation when the vessel is damaged.[3] In contrast, endothelial cells and monocytes do not express TF under physiological conditions; as a consequence, there is no appreciable contact of cellular TF with the circulating blood. In response to various stimuli, however, TF expression and activity can be induced in these cells as well.

The coagulation cascade is initiated as soon as TF comes into contact with circulating activated factor VII (VIIa), resulting in the TF-FVIIa complex (Figure 1). Alternatively, TF can bind inactive factor VII and form the TF-FVII complex, which is converted to TF-FVIIa by FVIIa or already formed TF-FVIIa. The TF-VIIa complex activates factor IX, which in turn activates factor X; alternatively, factor X is directly converted to factor Xa by TF-FVIIa. In complex with factor Va and calcium, Factor Xa catalyzes the conversion of prothrombin to thrombin, thereby leading to fibrin formation, platelet activation, and, ultimately, generation of a thrombus. Several of these activated proteases, including factor IXa, factor Xa, thrombin, and the TF-VIIa complex itself, can convert factor VII to VIIa in an auto-feedback loop.

In addition to its well-established role in coagulation, TF participates in other cellular processes. It is involved in migration and proliferation of vascular smooth muscle cells.[4] Furthermore, the development of embryonic blood vessels is critically dependent on TF because mice lacking TF die beyond embryonic day 8.5 secondary to abnormal circulation from yolk sac to embryo.[5] TF has also been observed to promote tumor neovascularization and metastasis.

Over the last years, our knowledge of the molecular mechanisms regulating TF expression as well as its biological

From Cardiovascular Research, Physiology Institute, University of Zürich, Center for Integrative Human Physiology, University of Zürich, and Cardiology, Cardiovascular Center, University Hospital Zürich, Zürich, Switzerland.

Correspondence to Felix C. Tanner, MD, Cardiovascular Research, Physiology Institute, University of Zurich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. E-mail felix.tanner@access.unizh.ch

© 2006 American Heart Association, Inc.

Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/CIRCULATIONAHA.105.567297**

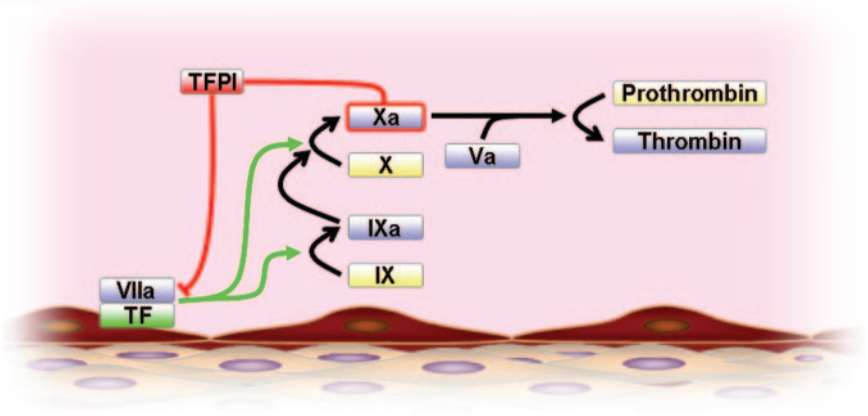


Figure 1. Tissue factor: A key regulator of coagulation. Tissue factor (TF) is a key initiator of the coagulation cascade. Formation of a complex with factor VIIa (FVIIa) leads to activation of factor IX (FIX) and factor X (FX), resulting in thrombin generation and, ultimately, clot formation. Tissue factor pathway inhibitor (TFPI), the endogenous inhibitor of TF activity, is synthesized and secreted mainly by endothelial cells. TFPI binds to FXa and thereby inhibits TF/FVIIa activity.

action in vascular cells has greatly advanced and is delineated in this section.

Endothelial Cells

Endothelial TF is induced by cytokines such as tumor necrosis factor-α (TNF-α),[6] interleukin-1β,[7] or CD40 ligand,[8] by biogenic amines such as serotonin[9] or histamine,[10] and by mediators such as thrombin, oxidized LDL, or vascular endothelial growth factor (Figure 2).[11–13] Despite their diversity, most of these mediators share similar signal transduction pathways regulating TF induction. The MAP kinases p38, p44/42 (ERK), and c-*jun* terminal NH_2-kinase (JNK) are involved in TNF-α–induced, histamine-induced, and thrombin-induced TF expression,[6,10,12,14] whereas the effect of vascular endothelial growth factor (VEGF) is only mediated by p38 and ERK.[14] TNF-α and VEGF are known to induce TF expression through activation of protein kinase C as well.[14] These signal transduction molecules stimulate the TF promoter by activating transcription factors such as AP-1, nuclear factor (NF)-κB, and EGR-1,[8,14,15] ultimately resulting in upregulation of TF mRNA.[13,15–17] Regulation of the TF promoter is reviewed in detail elsewhere.[15]

Unlike MAP kinases or protein kinase C, the PI3-kinase pathway negatively regulates endothelial TF expression; as a consequence, inhibition of PI3-kinase or its downstream mediators increases TF expression in response to TNF-α, histamine, thrombin, and VEGF.[12,16,18,19] The mechanism of the enhanced endothelial TF expression on PI3-kinase inhibition has not yet been elucidated in detail. Downstream targets of PI3-kinase such as the mammalian target of rapamycin are known to be involved in the regulation of protein translation, which may at least in part account for their effect on TF expression.[16] In one study, VEGF-induced p38 activation was enhanced when PI3-kinase was blocked by wortmannin[19]; hence, cross-talk to MAP kinases may explain the inhibitory role of the PI3-kinase pathway on VEGF-induced TF expression. In contrast, such cross-talk is not observed in response to thrombin,[16] indicating that the molecular events underlying the effect of PI3-kinase on endothelial TF expression differ with the particular stimulus involved.

The extent of TF protein induction in vascular cells does not always correlate well with TF activity.[10,11] One possible explanation is the concomitant secretion of tissue factor pathway inhibitor (TFPI), the endogenous inhibitor of TF. Another possible reason is the distribution of TF into several cellular compartments.[11,20] Biologically active TF is indeed located at the cell surface, whereas intracellular TF constitutes a pool that is only released on cell damage. A combination of TNF-α and VEGF favors cell surface over intracel-

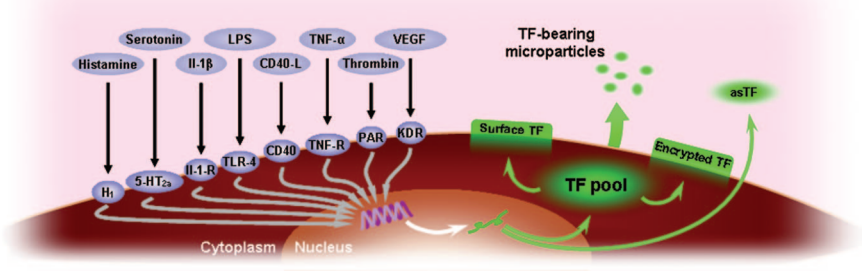


Figure 2. Induction of tissue factor expression and activity. Induction of tissue factor (TF) is exemplified in an endothelial cell. Various mediators induce TF expression through activation of their receptors. Induction of TF primarily occurs at the transcriptional level, resulting in an increase in TF mRNA and, eventually, TF protein expression. TF is distributed in three cellular pools as cytoplasmatic TF, surface TF, and encrypted TF. Moreover, TF-containing microparticles are released from the cell. Alternative splicing results in a soluble secreted form of TF (asTF). IL-1β indicates interleukin-1β; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor; HB_{1B}, histamine HB_{1B}-receptor; 5-HTB_{2aB}, 5-hydroxytryptamineB_{2aB} receptor; IL1-R, interleukin-1 receptor; TLR-4, toll-like receptor 4; PAR, protease-activated receptor; KDR, VEGF receptor-2.

lular distribution as compared with stimulation with either agonist alone, suggesting a complex regulation of the cellular distribution of TF.[11] Discrepancies between TF protein expression and activity can further be accounted for by the induction of a functionally inactive form of TF at the cell surface, termed latent or encrypted tissue factor. Expression of encrypted TF enables a cell to rapidly increase TF activity in response to certain stimuli without the need for de novo protein synthesis because de-encryption of TF has been observed secondary to changes in intracellular calcium levels, alterations in membrane phosphatidylserine expression, or modifications in the quaternary structure of TF.[21] Hence, the relative contribution of TF protein induction, cellular localization, and structural modification appears to determine the net procoagulant effect elicited by a given mediator.

Vascular Smooth Muscle Cells

In normal arteries, low levels of TF are found in the medial layer.[3] Similarly, vascular smooth muscle cells in culture express low basal levels of TF.[10,20,22,23] Mediators such as TNF-α, CD40 ligand, histamine, thrombin, endotoxin, and PDGF-BB as well as aggregated LDL and lysophosphatidic acid induce TF expression in vascular smooth muscle cells.[10,20,22,24–28] In general, the extent of inducibility in response to these mediators appears to be lower than that observed in endothelial cells or monocytes. Relatively few studies have investigated the molecular mechanisms regulating TF expression in vascular smooth muscle cells. Similar to endothelial cells, an involvement of MAP kinases and PI3-kinase has been observed. In human vascular smooth muscle cells, p38 and PI3-kinase mediate thrombin-induced TF expression.[26] In rat vascular smooth muscle cells, ERK is involved in TF expression to lysophosphatidic acid[28] and PDGF-BB,[25] whereas p38 and PI3-kinase do not play a role. Hence, the effect of MAP kinases and the PI3 kinase pathway appears to depend on the particular stimulus and/or the species involved.

Similar to endothelial cells, induction of vascular smooth muscle cell TF expression is only to some extent reflected in an increased TF activity.[20] Consistent with this observation, vascular smooth muscle cell TF is found in three cellular pools, namely as surface TF, encrypted TF, and intracellular TF.[20,29]

Monocytes and Macrophages

Monocytes, like endothelial cells, show very little to no basal expression of TF. Its expression can, however, be induced by inflammatory stimuli such as C-reactive protein[30] or CD40 ligand.[31] PDGF-BB, angiotensin II, and oxidized LDL[32,33] have also been observed to induce TF in monocytes; yet one of the most extensively studied stimuli in this cell type is endotoxin.[15,34] p38, ERK, and JNK are all involved in lipopolysaccharide-induced monocyte TF expression[34] leading to nuclear translocation of the transcription factors EGR-1, c-*Fos*/c-*Jun*, and c-*Rel*/p65. Ultimately, binding of these transcription factors to the EGR-1, AP-1, and NF-κB sites of the TF promoter mediates the endotoxin-induced increase in TF mRNA transcription.[15] Similar to endothelial cells, the PI3 kinase pathway exerts an inhibitory influence on TF induction in this cell type, and at least part of this action occurs through cross-talk with MAP kinases.[34]

Type 1 (TH_1) but not type 2 helper T cells (TH_2) secrete mainly proinflammatory mediators such as TNF-α and interferon (IFN)-γ, which are involved in macrophage activation.[35] Cytokines derived from TH_1 cells as well as cell-to-cell contact with TH_1 cells induces TF expression in monocytes.[36] Transformation of monocyte-derived macrophages into foam cells results in increased TF expression as well.[37] In contrast, TH_2-derived mediators such as IL-4, IL-10, and IL-13 prevent TH_1-induced TF expression.[36] Thus, consistent with their role in macrophage activation and atherogenesis, proinflammatory stimuli released from TH_1 cells upregulate TF expression, whereas cytokines derived from TH_2 cells inhibit this effect.

Blood-Borne Tissue Factor

Tissue factor is not only present in vascular cells or leukocytes but can also be detected in the bloodstream, referred to as circulating or blood-borne TF.[38] This form of TF is mainly associated with microparticles[39] originating from endothelial cells, vascular smooth muscle cells, leukocytes, or platelets.[22,40] In addition, TF containing microparticles are released from atherosclerotic plaques.[39]

Monocytes and platelets are known to exchange microparticle-bound TF.[41] Because megakaryocytes, the bone marrow sedentary precursors of platelets, do not express TF, it is likely that this exchange represents a mechanism through which platelets are loaded with TF. In addition to carrying microparticle-derived TF, activated platelets induce tissue factor expression in human endothelial and smooth muscle cells, presumably by releasing soluble mediators such as serotonin and PDGF.[42] Aggregating platelets thus induce a positive feedback loop that enhances local TF concentrations through two mechanisms and may be important for thrombus formation and/or propagation.

Recently, an alternatively spliced form of TF has been discovered, which is soluble, circulates in the blood, and exhibits procoagulant activity.[2] Cytokines stimulate its expression in and release from endothelial cells.[43] Alternatively spliced TF is not bound to microparticles and appears to represent a distinct form of circulating TF; as such, it may have an important role in thrombus propagation.[43] Indeed, soluble TF may be particularly important in this context, as vessel wall–associated TF, being separated from the bloodstream by the freshly formed thrombus, may be prevented from contributing to thrombus growth.[44]

These studies on blood-borne TF imply that activation of coagulation, contrary to traditional belief, may be initiated and propagated without contact of the blood to the extravascular space. The importance of blood-borne versus vessel wall-associated TF is currently a subject of debate.[44–46] One study described that TF from leukocyte-derived microparticles importantly contributes to thrombus propagation in an animal model of thrombosis,[44] whereas another one identified vessel wall–derived TF as the primary mediator driving thrombus formation after vascular injury.[45] It is also controversial whether physiological concentrations of circulating TF can exhibit clot-forming activity in vivo.[47] Thus, the

relative contribution of soluble TF, microparticle-bound TF, and vessel wall–associated TF to initiation and propagation of thrombosis requires further study.

Tissue Factor in Cardiovascular Diseases

Tissue factor has been implicated in the pathogenesis of several cardiovascular disorders. The following section summarizes its involvement in cardiovascular risk factors such as hypertension, diabetes, dyslipidemia, and smoking; its role in atherosclerosis and acute coronary syndromes is discussed as well.

Cardiovascular Risk Factors

Tissue factor plasma antigen is elevated in hypertensive subjects as compared with normotensive control subjects and can be lowered by different classes of antihypertensive drugs.[48] Of particular interest is the observation that angiotensin II induces TF expression in monocytes, endothelial cells, and vascular smooth muscle cells[24,32] through the angiotensin II type I receptor (AT-I).[32] Consistently, both ACE inhibitors[49] and AT-I receptor blockers[50] reduce TF plasma activity in hypertensive patients. ACE inhibitors also reduce endotoxin-induced TF expression in monocytes,[51] suggesting a pleiotropic effect of this class of drugs.

High glucose concentrations increase thrombin-induced TF expression in human endothelial cells.[52] Similarly, glucose intake upregulates TF expression in monocytes of healthy humans. Hyperglycemia leads to the formation of advanced glycation end-products (AGE), which induce TF expression in endothelial cells through the receptor for advanced glycation end-products (RAGE) and activation of NF-κB.[53,54] Consistent with this observation, diabetic ApoE$^{-/-}$ mice display increased vascular expression of RAGE and TF[55]; moreover, blockade of RAGE suppresses TF levels in the aorta of these mice, which may have interesting implications for the pathogenesis and treatment of diabetic vasculopathy.[55] In diabetic patients, increased TF plasma levels are measured even without overt coronary artery disease,[56] and TF levels are reduced by improving glycemic control.[57] In obese nondiabetic subjects, insulin reduces both monocyte TF expression and TF plasma levels, which occurs due to a reduced activation of the proinflammatory transcription factor EGR-1, suggesting that insulin exerts its beneficial effects not only through improving hyperglycemia.[58]

Oxidized LDL increases TF expression in endothelial cells,[13] monocytes, and macrophages,[33] whereas reconstituted HDL inhibits thrombin-induced endothelial TF expression.[18] Consistent with these observations, patients with elevated LDL levels display raised TF plasma activity.[57] HMG-CoA reductase inhibitors (statins), the most widely used drugs for the treatment of hypercholesterolemia, reduce TF expression in monocytes, endothelial cells, and vascular smooth muscle cells.[12,59] In apoE knockout mice, simvastatin inhibits TF expression in advanced atherosclerotic lesions independent of plasma lipid levels.[60] Hence, the reduction of TF expression by statins is at least in part related to the pleiotropic antiinflammatory effects of this class of drugs. Consistent with this interpretation, endotoxin-induced TF expression is blunted after administration of simvastatin to healthy humans.[61] Fibric acid derivatives are another class of drugs used to treat patients with dyslipidemia. Through activation of the peroxisome proliferator-activated receptor–alpha (PPAR-α), these drugs reduce TF expression in human monocytes and macrophages, which may in part be responsible for their beneficial effect in patients with cardiovascular diseases.[62]

Exposure of apoE knockout mice to cigarette smoke results in an increased TF expression in atherosclerotic plaques as compared with mice breathing filtered room air.[63] Cigarette smoking is associated with increased TF plasma levels in humans as well; indeed, a strong correlation is observed between the number of cigarettes smoked and TF plasma levels.[57]

These data indicate that TF may be involved in the proatherosclerotic effect of cardiovascular risk factors. It cannot be ruled out, however, that some of the observed elevations in TF plasma levels occur secondary to TF release from already established atherosclerotic plaques.

Atherosclerosis

Monocytes infiltrate the intimal layer and then transform into macrophages and foam cells, which represents a hallmark of the inflammatory nature of atherosclerosis.[35] In this inflammatory environment, cytokines such as TNF-α and interleukins are released and induce expression of TF. During the early stages of atherogenesis, enhanced TF expression is observed in monocytes[3]; at later stages, TF expression is also detected in foam cells, endothelial cells, and smooth muscle cells.[3,64] TF is present in the necrotic core of plaques as well, predominantly associated with microparticles derived from perishing foam cells, macrophages, or lymphocytes.[39,64] Such microparticles indeed contain the major part of the TF activity in atherosclerotic plaques.[39] Consistent with these observations, TF expression is closely associated with apoptosis of macrophages in lipid-rich plaques,[65] which adds to the evidence that not only inflammation but also apoptosis can determine plaque thrombogenicity.

Increasing size of atherosclerotic plaques can lead to vascular stenosis, which is associated with enhanced shear forces promoting endothelial TF expression. In most instances, however, it is not the degree of luminal narrowing, but rather the composition of the plaque which determines the clinical course of events. Lipid-rich plaques with a thin cap, a large lipid core, extensive macrophage infiltration, and abundant TF expression are more prone to rupture than collagen-rich, fibrous plaques. Rupture of an atherosclerotic plaque exposes its highly procoagulant content to the circulating blood; thereby, TF-laden macrophages as well as TF-containing microparticles originating from the necrotic core initiate thrombus formation and related complications such as acute myocardial infarction (Figure 3).

Besides activation of the coagulation cascade, TF is involved in other pathogenetic events occurring during the formation of atherosclerotic lesions. TF is the receptor for factor VIIa and as such mediates responses such as migration[66] and proliferation of vascular smooth muscle cells.[67] These processes are importantly involved in vascular remodeling, because vascular smooth muscle cells migrate into and proliferate within the neointima of injured vessels. The effect

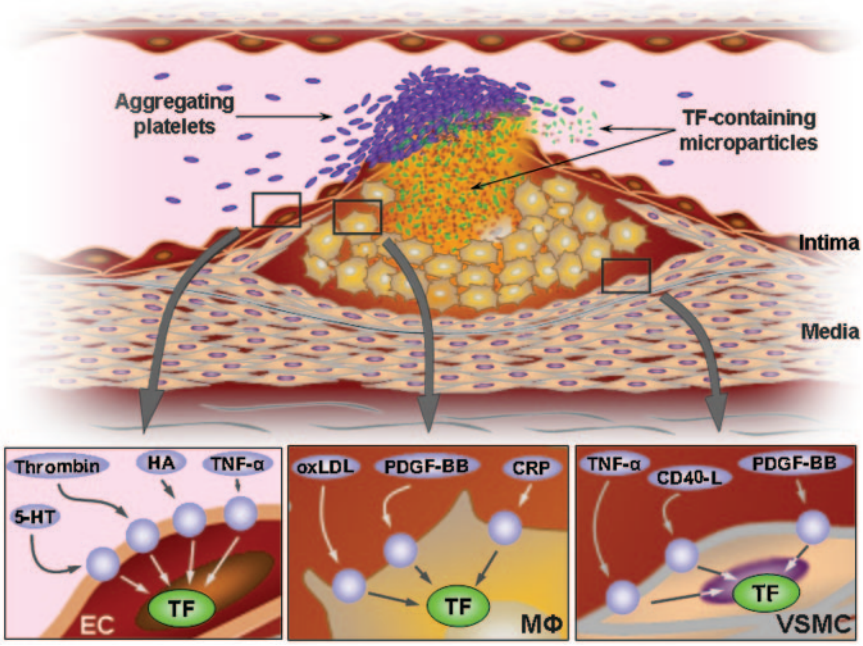


Figure 3. Tissue factor in the atherosclerotic plaque. In the inflammatory environment of atherosclerotic plaques, tissue factor (TF) is present at high levels in endothelial cells, vascular smooth muscle cells, macrophages/foam cells, and in the necrotic core. TF induction is exemplified by selected mediators in endothelial cells (EC, left panel), macrophages (Mφ, middle panel), and vascular smooth muscle cells (VSMC, right panel). On plaque rupture, highly procoagulant material including TF-containing microparticles is released into the blood, leading to rapid initiation of coagulation, platelet aggregation, and, ultimately, thrombus formation with vessel occlusion.

of the TF/FVIIa complex on migration and proliferation of vascular smooth muscle cells is critically dependent on the cytoplasmic domain of TF, as vascular remodeling in response to injury is reduced in mice lacking this domain.[68] Recruitment of microvessels into atherosclerotic plaques can lead to plaque progression by contributing to plaque destabilization and rupture.[35] As TF is involved in angiogenesis,[5] it may also play a role in plaque neovascularization and thereby promote plaque destabilization. Thus, evidence is emerging that TF may not only be involved in atherogenesis by eliciting thrombosis but also by direct actions on vascular remodeling and plaque progression or instability.

Acute Coronary Syndrome and Percutaneous Coronary Intervention

Increased levels of TF antigen and activity are detected in atherectomy specimens from patients with unstable angina or myocardial infarction as compared with those with stable angina.[69] In acute coronary syndromes, plasma concentrations of inflammatory cytokines such as TNF-α and interleukins are indeed increased at the site of coronary artery occlusion to such an extent that TF is induced in vascular cells.[70] Hence, vascular cells as well as circulating leukocytes and aggregating platelets may be a source of the elevated levels of circulating TF measured in patients with acute coronary syndrome.[71] As plaque rupture leads to exposure of highly procoagulant plaque content to the circulation, it may also contribute to the elevated TF plasma levels.[39] Consistent with this interpretation, patients with unstable angina show higher TF plasma levels than those with stable angina,[72] and elevated TF plasma levels may even predict future cardiovascular events in patients with unstable angina.[72] Because a substantial number of patients with acute myocardial infarction have coronary artery thrombi on top of a superficial erosion, increased TF plasma levels in these patients may also originate from endothelial erosions of atherosclerotic lesions.[73] Interestingly, several polymorphisms of the TF gene are known, and recent data suggest that certain genetic variations in the TF gene as well as the TF promoter may be associated with a worse outcome in patients with acute coronary syndrome, possibly through increased monocyte TF expression.[74,75]

Selective cyclooxygenase-2 inhibitors (coxibs), rofecoxib in particular, have come under scrutiny as they are suspected of causing thrombotic complications in patients with cardiovascular diseases. This effect is believed to depend on inhibition of prostacyclin formation. Large-scale epidemiological studies have indeed suggested that rofecoxib intake is associated with an increased risk of myocardial infarction.[76] This association, however, is not observed with celecoxib, which may be related to inhibition of TF expression by this coxib in endothelial cells.[6] Given the importance of TF in the pathogenesis of acute coronary syndromes, this effect may account for the different occurrence of thrombotic complications between coxibs observed in epidemiological studies.[76] As neither rofecoxib nor the experimental coxib NS-398 inhibit endothelial TF expression, the effect of celecoxib appears to occur independent of cyclooxygenase inhibition; instead, it is mediated by decreasing TNF-α–induced JNK phosphorylation.[6]

Because platelets represent a major source of TF, it is conceivable that antiplatelet drugs, which are used for treating acute coronary syndromes, reduce TF expression as well as TF plasma levels. Indeed, the ADP receptor antagonist clopidogrel reduces TF expression in the ischemic coronary artery in an animal model of acute myocardial infarction.[77] Moreover, as interaction with platelets induces TF expression in monocytes, the GPIIb/IIIa antagonist abciximab suppresses monocyte TF expression and activity by reducing platelet-monocyte cross-talk; likewise, abciximab reduces monocyte TF expression in patients undergoing carotid angioplasty with stenting.[78] Aspirin, however, inhibits endotoxin-induced tran-

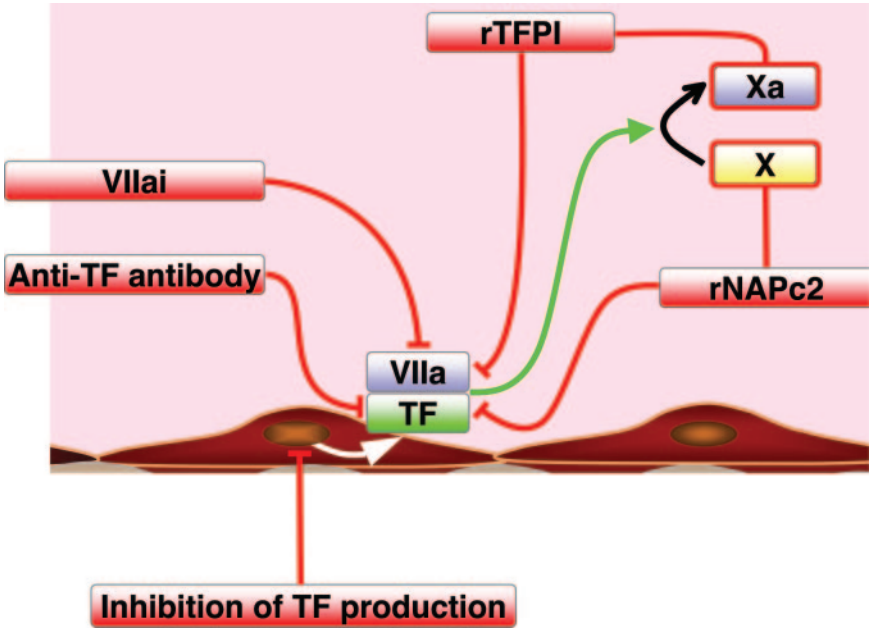


Figure 4. Therapeutic approaches. Several therapeutic strategies have been developed to specifically interfere with the action of tissue factor (TF). Molecular approaches such as ribozymes or antisense oligonucleotides specifically inhibit TF production. Monoclonal or polyclonal anti-TF antibodies directly target and inactivate the TF protein. Site-inactivated factor VIIa (FVIIai) binds to TF but lacks catalytic activity for conversion of factor X (FX) or factor IX (FIX). Recombinant tissue factor pathway inhibitor (rTFPI) interferes with the activity of the TF/FVIIa complex by binding to the active site of factor Xa (FXa), leading to formation of a quaternary inhibitory complex with TF/FVIIa. Similarly, recombinant nematode anticoagulant protein c2 (rNAPc2) interferes with the TF/FVIIa complex by binding to FXa or FX before formation of a quarternary inhibitory complex with TF/FVIIa.

scriptional activation of TF expression in monocytes and may thus reduce TF plasma activity by direct actions on the regulation of TF expression as well.[79] In contrast to these agents, long-term treatment with the oral anticoagulant warfarin increases soluble TF levels, probably as the result of decreased TF consumption.[80]

Percutaneous coronary intervention is the preferred treatment for patients presenting with acute myocardial infarction. It is unclear whether or not TF plasma levels are increased after percutaneous coronary intervention, as some groups found an increase whereas others did not.[81] It is conceivable that plaque dissection caused by balloon dilation may lead to exposure of plaque content to the bloodstream and thereby increase TF plasma levels. In the reperfusion phase, oxygen-derived free radicals were observed to mediate TF induction, whereas continuous inflammatory alterations of the atherosclerotic coronary arteries may increase TF levels at later stages.[82] If present, increased TF levels have a negative prognostic value with regard to the development of restenosis after percutaneous coronary angioplasty with or without stenting.[81] This effect may well be related to the promigratory and proproliferative action of TF on vascular smooth muscle cells, which are known to contribute to the development of restenosis.[4,66,67]

Drug-eluting stents are covered with pharmacological agents, which, once released into the coronary artery after stent deployment, inhibit vascular smooth muscle cell proliferation and thereby restenosis. In contrast to reduced restenosis rates, however, the frequency of stent thromboses has not decreased with drug-eluting stents as compared with bare metal stents.[83] Rapamycin, which is used for stent coating, increases endothelial TF expression, suggesting a potential role for this drug in the development of subacute stent thrombosis.[16] As this effect on TF expression is not observed with FK-506, another agent used on drug-eluting stents, application of FK-506-eluting stents may provide a more favorable environment for the development of in-stent thrombosis.[16] However, additional studies are needed to assess the implications of these findings in vivo. Platelet activation is a crucial event in the pathogenesis of thrombus formation. The use of platelet receptor antagonists such as clopidogrel has indeed reduced the incidence of stent thrombosis, whereas withdrawal of antiplatelet therapy favors thrombus formation.[83] Clopidogrel inhibits the release of TF from aggregating platelets, which is of particular importance, as platelet aggregation and secretion are increased in human platelets treated with rapamycin.[84] Therefore, it will be of great interest to study the dynamic interaction between rapamycin and platelet activation as well as the spatio-temporal pattern of TF expression in the arterial wall after deployment of drug-eluting stents.

Therapeutic Implications

Various agents have been developed to specifically interfere with the action of TF and the TF/FVIIa complex (Figure 4). In contrast to classic antithrombotic drugs, these agents target the first steps of coagulation while leaving the downstream effectors intact. In addition, these drugs can interfere with promigratory or proproliferative effects of TF. In this section, we will focus on recently developed drugs specifically targeting TF, whereas nonspecific inhibitors will not be discussed.

Inhibition of TF Synthesis

A hairpin ribozyme, which destroys TF mRNA, abrogated induction of TF protein expression and activity in vascular smooth muscle cells.[85] Similarly, antisense oligonucleotides, hybridizing to their complementary target mRNA and thereby preventing translation, inhibited TF induction in monocytes.[86] An alternative approach takes advantage of curcumin, a naturally occurring pigment suppressing the activation of the transcription factors Egr-1, AP-1, and NF-κB, and resulting in inhibition of TNF-α–induced endothelial TF induction.[87]

Although novel and intriguing, the clinical applicability of these approaches has not yet been examined.

Direct Inhibition of TF Action

Different antibody preparations directed against the TF antigen (TF Ab) have been tested in vivo. In a rabbit carotid artery thrombosis model, a monoclonal anti–TF Ab inhibited thrombus formation and shortened tissue plasminogen activator lysis time.[88] Similarly, administration of a monoclonal anti–TF Ab reduced infarct size in a rabbit coronary artery ligation model as the result of reduced chemokine expression and leukocyte infiltration.[89] In humans, a polyclonal anti–TF Ab reduced thrombogenicity of disrupted atherosclerotic plaques by impairing platelet and fibrin deposition.[90] In a recent dose-escalating trial, a chimeric monoclonal anti–TF Ab potently inhibited thrombin formation in patients with stable coronary artery disease.[91]

An alternative approach consists of a mutant form of TF, which binds to factor VIIa but exhibits reduced catalytic activity.[92] In a rabbit model of arterial thrombosis, this protein displayed potent antithrombotic properties while causing less bleeding than heparin.[92] Another mutated form of TF exhibited antithrombotic properties in a guinea pig model of recurrent arterial thrombosis.[93]

Active-Site Inactivated Factor VIIa

Active-site inactivated factor VIIa (FVIIai) binds to TF but lacks catalytic activity; as such, it competes for TF with the physiologically occurring form of factor VIIa. Several animal studies examined the effect of FVIIai in vivo, demonstrating decreased recurrent arterial thrombosis, reduced infarct size, and improved no-reflow phenomenon; after administration of a single dose, the effect of FVIIai was long-lasting without affecting systemic hemostatic parameters.[94] Application of a structurally different but functionally similar site-inactivated factor VIIa compound reduced restenosis in a femoral artery injury model,[95] which may in part be due to the fact that TF as well as several downstream coagulation factors stimulate vascular smooth muscle cell migration and proliferation.[66,67]

Recombinant Tissue Factor Pathway Inhibitor

Tissue factor activity is counterbalanced by its endogenous inhibitor, TFPI. TFPI interferes with activity of the TF/FVIIa complex by binding to the active site of FXa leading to formation of a quaternary complex with TF/FVIIa. Under physiological conditions, TFPI is mainly synthesized and released by endothelial cells. In animal models, recombinant TFPI (rTFPI) reduced fibrin deposition and neointimal thickening after balloon injury.[96] In human atherosclerotic arteries, rTFPI diminished plaque thrombogenicity by inhibiting platelet and fibrin deposition.[90]

Adenoviral gene transfer represents an alternative approach for application of a recombinant protein.[97] Adenoviral overexpression of TFPI in injured arteries inhibited recurrent thrombosis induced by shear stress without affecting systemic coagulation parameters.[98] Similarly, overexpression of TFPI reduced thrombogenicity as well as vascular remodeling of balloon-injured atherosclerotic arteries.[99]

Based on these observations, clinical studies have investigated the safety and efficacy of rTFPI. Initial trials revealed promising results in various settings; a double-blinded, randomized, phase III trial in patients with severe sepsis and mild coagulopathy, however, failed to show a clinical benefit of rTFPI, whereas the frequency of severe adverse events with bleeding was increased.[100] Further studies are needed to definitively assess the safety and efficacy of rTFPI as a therapeutic principle.

Nematode Anticoagulant Protein c2

Nematode anticoagulant protein c2 (NAPc2) is isolated from the saliva of the hookworm *Ancylostoma caninum*; it interferes with TF activity by binding to factor Xa or factor X before formation of a quarternary inhibitory complex with TF/FVIIa. Several studies in primates as well as first studies in humans revealed very promising results; in a phase II clinical trial, rNAPc2 indeed appeared safe and effective in preventing thrombin generation during coronary angioplasty in combination with aspirin, clopidogrel, and heparin.[101]

Summary and Conclusions

Over the last years, major advances have been made in elucidating the molecular regulation of TF expression. Various cytokines, growth factors, and biogenic amines have been recognized to induce TF expression in endothelial cells, vascular smooth muscle cells, and monocytes. Signal transduction mechanisms specific for both the cell type and the stimulus involved regulate TF induction and cellular distribution. In addition to cellular TF, an important role of blood-borne TF is emerging; at present, however, the relative contribution of vessel wall–associated versus blood-borne TF to thrombus formation and/or propagation is debated.

Patients with cardiovascular risk factors such as hypertension, diabetes, dyslipidemia, and smoking have elevated plasma levels of TF, which may be involved in the proatherosclerotic effect of such risk factors. Moreover, TF expression is upregulated in the inflammatory environment of atherosclerotic plaques, and large amounts of TF are released during plaque rupture, leading to thrombus formation and elevated TF plasma levels in patients with unstable angina and acute coronary syndromes. As TF simulates vascular smooth muscle cell migration and proliferation, it may promote atherogenesis and restenosis not only by initiating thrombosis but also by direct actions on vascular remodeling and plaque progression.

Several promising therapeutic strategies have been developed for targeting the action of TF including antibodies against TF, site-inactivated factor VIIa, recombinant TFPI, and recombinant NAPc2. Despite recent setbacks with application of rTFPI, interfering with the TF pathway appears to be an attractive target for the treatment of cardiovascular diseases.

Acknowledgments

Our research in this area was supported by the Swiss National Science Foundation (grant 3200B0–102232/1 to F.C.T. and grant 3100-068118.02/1 to T.F.L.), the Bonizzi-Theler Foundation, the Hartmann-Müller Foundation, the Herzog-Egli Foundation, the Olga Mayenfisch Foundation, and the Swiss Heart Foundation.

Disclosures

None.

References

1. Mackman N, Morrissey JH, Fowler B, Edgington TS. Complete sequence of the human tissue factor gene, a highly regulated cellular receptor that initiates the coagulation protease cascade. *Biochemistry*. 1989;28:1755–1762.
2. Bogdanov VY, Balasubramanian V, Hathcock J, Vele O, Lieb M, Nemerson Y. Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. *Nat Med*. 2003;9:458–462.
3. Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proc Natl Acad Sci U S A*. 1989;86:2839–2843.
4. Pyo RT, Sato Y, Mackman N, Taubman MB. Mice deficient in tissue factor demonstrate attenuated intimal hyperplasia in response to vascular injury and decreased smooth muscle cell migration. *Thromb Haemost*. 2004;92:451–458.
5. Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Van Vlaenderen I, Demunck H, Kasper M, Breier G, Evrard P, Muller M, Risau W, Edgington T, Collen D. Role of tissue factor in embryonic blood vessel development. *Nature*. 1996;383:73–75.
6. Steffel J, Hermann M, Greutert H, Gay S, Luscher TF, Ruschitzka F, Tanner FC. Celecoxib decreases endothelial tissue factor expression through inhibition of c-Jun terminal NH2 kinase phosphorylation. *Circulation*. 2005;111:1685–1689.
7. Napoleone E, Di Santo A, Lorenzet R. Monocytes upregulate endothelial cell expression of tissue factor: a role for cell-cell contact and cross-talk. *Blood*. 1997;89:541–549.
8. Bavendiek U, Libby P, Kilbride M, Reynolds R, Mackman N, Schonbeck U. Induction of tissue factor expression in human endothelial cells by CD40 ligand is mediated via activator protein 1, nuclear factor kappa B, and Egr-1. *J Biol Chem*. 2002;277:25032–25039.
9. Kawano H, Tsuji H, Nishimura H, Kimura S, Yano S, Ukimura N, Kunieda Y, Yoshizumi M, Sugano T, Nakagawa K, Masuda H, Sawada S, Nakagawa M. Serotonin induces the expression of tissue factor and plasminogen activator inhibitor-1 in cultured rat aortic endothelial cells. *Blood*. 2001;97:1697–1702.
10. Steffel J, Akhmedov A, Greutert H, Luscher TF, Tanner FC. Histamine induces tissue factor expression: implications for acute coronary syndromes. *Circulation*. 2005;112:341–349.
11. Camera M, Giesen PL, Fallon J, Aufiero BM, Taubman M, Tremoli E, Nemerson Y. Cooperation between VEGF and TNF-alpha is necessary for exposure of active tissue factor on the surface of human endothelial cells. *Arterioscler Thromb Vasc Biol*. 1999;19:531–537.
12. Eto M, Kozai T, Cosentino F, Joch H, Luscher TF. Statin prevents tissue factor expression in human endothelial cells: role of Rho/Rho-kinase and Akt pathways. *Circulation*. 2002;105:1756–1759.
13. Drake TA, Hannani K, Fei HH, Lavi S, Berliner JA. Minimally oxidized low-density lipoprotein induces tissue factor expression in cultured human endothelial cells. *Am J Pathol*. 1991;138:601–607.
14. Mechtcheriakova D, Schabbauer G, Lucerna M, Clauss M, De Martin R, Binder BR, Hofer E. Specificity, diversity, and convergence in VEGF and TNF-alpha signaling events leading to tissue factor up-regulation via EGR-1 in endothelial cells. *FASEB J*. 2001;15:230–242.
15. Mackman N. Regulation of the tissue factor gene. *Thromb Haemost*. 1997;78:747–754.
16. Steffel J, Latini RA, Akhmedov A, Zimmerman D, Zimmerling P, Luscher TF, Tanner FC. Rapamycin, but not FK-506, increases endothelial tissue factor expression: implications for drug-eluting stent design. *Circulation*. 2005;112:2002–2011.
17. Liu Y, Pelekanakis K, Woolkalis MJ. Thrombin and TNFalpha synergistically stimulate tissue factor expression in human endothelial cells: regulation through c-Fos and c-Jun. *J Biol Chem*. 2004;279:36142–36147.
18. Viswambharan H, Ming XF, Zhu S, Hubsch A, Lerch P, Vergeres G, Rusconi S, Yang Z. Reconstituted high-density lipoprotein inhibits thrombin-induced endothelial tissue factor expression through inhibition of RhoA and stimulation of phosphatidylinositol 3-kinase but not Akt/endothelial nitric oxide synthase. *Circ Res*. 2004;94:918–925.
19. Blum S, Issbruker K, Willuweit A, Hehlgans S, Lucerna M, Mechtcheriakova D, Walsh K, von der Ahe D, Hofer E, Clauss M. An inhibitory role of the phosphatidylinositol 3-kinase-signaling pathway in vascular endothelial growth factor-induced tissue factor expression. *J Biol Chem*. 2001;276:33428–33434.
20. Schecter AD, Giesen PL, Taby O, Rosenfield CL, Rossikhina M, Fyfe BS, Kohtz DS, Fallon JT, Nemerson Y, Taubman MB. Tissue factor expression in human arterial smooth muscle cells: TF is present in three cellular pools after growth factor stimulation. *J Clin Invest*. 1997;100:2276–2285.
21. Wolberg AS, Monroe DM, Roberts HR, Hoffman MR. Tissue factor de-encryption: ionophore treatment induces changes in tissue factor activity by phosphatidylserine-dependent and -independent mechanisms. *Blood Coagul Fibrinolysis*. 1999;10:201–210.
22. Llorente-Cortes V, Otero-Vinas M, Camino-Lopez S, Llampayas O, Badimon L. Aggregated low-density lipoprotein uptake induces membrane tissue factor procoagulant activity and microparticle release in human vascular smooth muscle cells. *Circulation*. 2004;110:452–459.
23. Xuereb JM, Sie P, Boneu B, Constans J. Up-regulation of tissue factor expression by platelet-derived growth factor in human vascular smooth muscle cells in culture: role of mitogen-activated protein kinase and effects of intracellular cyclic AMP. *Thromb Haemost*. 1997;78:1520–1526.
24. Taubman MB, Marmur JD, Rosenfield CL, Guha A, Nichtberger S, Nemerson Y. Agonist-mediated tissue factor expression in cultured vascular smooth muscle cells: role of Ca2+ mobilization and protein kinase C activation. *J Clin Invest*. 1993;91:547–552.
25. Kamimura M, Bea F, Akizawa T, Katus HA, Kreuzer J, Viedt C. Platelet-derived growth factor induces tissue factor expression in vascular smooth muscle cells via activation of Egr-1. *Hypertension*. 2004;44:944–951.
26. Herkert O, Diebold I, Brandes RP, Hess J, Busse R, Gorlach A. NADPH oxidase mediates tissue factor-dependent surface procoagulant activity by thrombin in human vascular smooth muscle cells. *Circulation*. 2002;105:2030–2036.
27. Schonbeck U, Mach F, Sukhova GK, Herman M, Graber P, Kehry MR, Libby P. CD40 ligation induces tissue factor expression in human vascular smooth muscle cells. *Am J Pathol*. 2000;156:7–14.
28. Cui MZ, Zhao G, Winokur AL, Laag E, Bydash JR, Penn MS, Chisolm GM, Xu X. Lysophosphatidic acid induction of tissue factor expression in aortic smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2003;23:224–230.
29. Penn MS, Patel CV, Cui MZ, DiCorleto PE, Chisolm GM. LDL increases inactive tissue factor on vascular smooth muscle cell surfaces: hydrogen peroxide activates latent cell surface tissue factor. *Circulation*. 1999;99:1753–1759.
30. Cermak J, Key NS, Bach RR, Balla J, Jacob HS, Vercellotti GM. C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. *Blood*. 1993;82:513–520.
31. Mach F, Schonbeck U, Bonnefoy JY, Pober JS, Libby P. Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of CD40: induction of collagenase, stromelysin, and tissue factor. *Circulation*. 1997;96:396–399.
32. He M, He X, Xie Q, Chen F, He S. Angiotensin II induces the expression of tissue factor and its mechanism in human monocytes. *Thromb Res*. Published online June 10, 2005. DOI: 10.1016/j.thromres.2005.04.033.
33. Wada H, Kaneko T, Wakita Y, Minamikawa K, Nagaya S, Tamaki S, Deguchi K, Shirakawa S. Effect of lipoproteins on tissue factor activity and PAI-II antigen in human monocytes and macrophages. *Int J Cardiol*. 1994;47:S21–S25.
34. Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J Biol Chem*. 2002;277:32124–32132.
35. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. 2005;352:1685–1695.
36. Del Prete G, De Carli M, Lammel RM, D'Elios MM, Daniel KC, Giusti B, Abbate R, Romagnani S. Th1 and Th2 T-helper cells exert opposite regulatory effects on procoagulant activity and tissue factor production by human monocytes. *Blood*. 1995;86:250–257.
37. Meisel SR, Xu XP, Edgington TS, Dimayuga P, Kaul S, Lee S, Fishbein MC, Cercek B, Shah PK. Differentiation of adherent human monocytes into macrophages markedly enhances tissue factor protein expression and procoagulant activity. *Atherosclerosis*. 2002;161:35–43.
38. Giesen PL, Rauch U, Bohrmann B, Kling D, Roque M, Fallon JT, Badimon JJ, Himber J, Riederer MA, Nemerson Y. Blood-borne tissue

factor: another view of thrombosis. *Proc Natl Acad Sci U S A*. 1999;96:2311–2315.
39. Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM, Tedgui A. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation*. 2000;101:841–843.
40. Schecter AD, Spirn B, Rossikhina M, Giesen PL, Bogdanov V, Fallon JT, Fisher EA, Schnapp LM, Nemerson Y, Taubman MB. Release of active tissue factor by human arterial smooth muscle cells. *Circ Res*. 2000;87:126–132.
41. Scholz T, Temmler U, Krause S, Heptinstall S, Losche W. Transfer of tissue factor from platelets to monocytes: role of platelet-derived microvesicles and CD62P. *Thromb Haemost*. 2002;88:1033–1038.
42. Cirillo P, Golino P, Calabro P, Ragni M, Forte L, Piro O, De Rosa S, Pacileo M, Chiariello M. Activated platelets stimulate tissue factor expression in smooth muscle cells. *Thromb Res*. 2003;112:51–57.
43. Szotowski B, Antoniak S, Poller W, Schultheiss HP, Rauch U. Procoagulant soluble tissue factor is released from endothelial cells in response to inflammatory cytokines. *Circ Res*. 2005;96:1233–1239.
44. Chou J, Mackman N, Merrill-Skoloff G, Pedersen B, Furie BC, Furie B. Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation. *Blood*. 2004;104:3190–3197.
45. Day SM, Reeve JL, Pedersen B, Farris DM, Myers DD, Im M, Wakefield TW, Mackman N, Fay WP. Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall. *Blood*. 2005;105:192–198.
46. Morrissey JH. Clotting hits the wall. *Blood*. 2005;105:3–4.
47. Butenas S, Bouchard BA, Brummel-Ziedins KE, Parhami-Seren B, Mann KG. Tissue factor activity in whole blood. *Blood*. 2005;105:2764–2770.
48. Felmeden DC, Spencer CG, Chung NA, Belgore FM, Blann AD, Beevers DG, Lip GY. Relation of thrombogenesis in systemic hypertension to angiogenesis and endothelial damage/dysfunction (a substudy of the Anglo-Scandinavian Cardiac Outcomes Trial [ASCOT]). *Am J Cardiol*. 2003;92:400–405.
49. Soejima H, Ogawa H, Yasue H, Kaikita K, Takazoe K, Nishiyama K, Misumi K, Miyamoto S, Yoshimura M, Kugiyama K, Nakamura S, Tsuji I. Angiotensin-converting enzyme inhibition reduces monocyte chemoattractant protein-1 and tissue factor levels in patients with myocardial infarction. *J Am Coll Cardiol*. 1999;34:983–988.
50. Koh KK, Chung WJ, Ahn JY, Han SH, Kang WC, Seo YH, Ahn TH, Choi IS, Shin EK. Angiotensin II type 1 receptor blockers reduce tissue factor activity and plasminogen activator inhibitor type-1 antigen in hypertensive patients: a randomized, double-blind, placebo-controlled study. *Atherosclerosis*. 2004;177:155–160.
51. Napoleone E, Di Santo A, Camera M, Tremoli E, Lorenzet R. Angiotensin-converting enzyme inhibitors downregulate tissue factor synthesis in monocytes. *Circ Res*. 2000;86:139–143.
52. Boeri D, Almus FE, Maiello M, Cagliero E, Rao LV, Lorenzi M. Modification of tissue-factor mRNA and protein response to thrombin and interleukin 1 by high glucose in cultured human endothelial cells. *Diabetes*. 1989;38:212–218.
53. Bierhaus A, Chevion S, Chevion M, Hofmann M, Quehenberger P, Illmer T, Luther T, Berentshtein E, Tritschler H, Muller M, Wahl P, Ziegler R, Nawroth PP. Advanced glycation end product-induced activation of NF-kappaB is suppressed by alpha-lipoic acid in cultured endothelial cells. *Diabetes*. 1997;46:1481–1490.
54. Bierhaus A, Illmer T, Kasper M, Luther T, Quehenberger P, Tritschler H, Wahl P, Ziegler R, Muller M, Nawroth PP. Advanced glycation end product (AGE)-mediated induction of tissue factor in cultured endothelial cells is dependent on RAGE. *Circulation*. 1997;96:2262–2271.
55. Kislinger T, Tanji N, Wendt T, Qu W, Lu Y, Ferran LJ Jr, Taguchi A, Olson K, Bucciarelli L, Goova M, Hofmann MA, Cataldegirmen G, D'Agati V, Pischetsrieder M, Stern DM, Schmidt AM. Receptor for advanced glycation end products mediates inflammation and enhanced expression of tissue factor in vasculature of diabetic apolipoprotein E-null mice. *Arterioscler Thromb Vasc Biol*. 2001;21:905–910.
56. Lim HS, Blann AD, Lip GY. Soluble CD40 ligand, soluble P-selectin, interleukin-6, and tissue factor in diabetes mellitus: relationships to cardiovascular disease and risk factor intervention. *Circulation*. 2004;109:2524–2528.
57. Sambola A, Osende J, Hathcock J, Degen M, Nemerson Y, Fuster V, Crandall J, Badimon JJ. Role of risk factors in the modulation of tissue factor activity and blood thrombogenicity. *Circulation*. 2003;107:973–977.
58. Aljada A, Ghanim H, Mohanty P, Kapur N, Dandona P. Insulin inhibits the pro-inflammatory transcription factor early growth response gene-1 (Egr)-1 expression in mononuclear cells (MNC) and reduces plasma tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) concentrations. *J Clin Endocrinol Metab*. 2002;87:1419–1422.
59. Brandes RP, Beer S, Ha T, Busse R. Withdrawal of cerivastatin induces monocyte chemoattractant protein 1 and tissue factor expression in cultured vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2003;23:1794–1800.
60. Bea F, Blessing E, Shelley MI, Shultz JM, Rosenfeld ME. Simvastatin inhibits expression of tissue factor in advanced atherosclerotic lesions of apolipoprotein E deficient mice independently of lipid lowering: potential role of simvastatin-mediated inhibition of Egr-1 expression and activation. *Atherosclerosis*. 2003;167:187–194.
61. Steiner S, Speidl WS, Pleiner J, Seidinger D, Zorn G, Kaun C, Wojta J, Huber K, Minar E, Wolzt M, Kopp CW. Simvastatin blunts endotoxin-induced tissue factor in vivo. *Circulation*. 2005;111:1841–1846.
62. Marx N, Mackman N, Schonbeck U, Yilmaz N, Hombach V, Libby P, Plutzky J. PPARalpha activators inhibit tissue factor expression and activity in human monocytes. *Circulation*. 2001;103:213–219.
63. Matetzky S, Tani S, Kangavari S, Dimayuga P, Yano J, Xu H, Chyu KY, Fishbein MC, Shah PK, Cercek B. Smoking increases tissue factor expression in atherosclerotic plaques: implications for plaque thrombogenicity. *Circulation*. 2000;102:602–604.
64. Thiruvikraman SV, Guha A, Roboz J, Taubman MB, Nemerson Y, Fallon JT. In situ localization of tissue factor in human atherosclerotic plaques by binding of digoxigenin-labeled factors VIIa and X. *Lab Invest*. 1996;75:451–461.
65. Hutter R, Valdiviezo C, Sauter BV, Savontaus M, Chereshnev I, Carrick FE, Bauriedel G, Luderitz B, Fallon JT, Fuster V, Badimon JJ. Caspase-3 and tissue factor expression in lipid-rich plaque macrophages: evidence for apoptosis as link between inflammation and atherothrombosis. *Circulation*. 2004;109:2001–2008.
66. Sato Y, Asada Y, Marutsuka K, Hatakeyama K, Sumiyoshi A. Tissue factor induces migration of cultured aortic smooth muscle cells. *Thromb Haemost*. 1996;75:389–392.
67. Cirillo P, Cali G, Golino P, Calabro P, Forte L, De Rosa S, Pacileo M, Ragni M, Scopacasa F, Nitsch L, Chiariello M. Tissue factor binding of activated factor VII triggers smooth muscle cell proliferation via extracellular signal-regulated kinase activation. *Circulation*. 2004;109:2911–2916.
68. Ott I, Weigand B, Michl R, Seitz I, Sabbari-Erfani N, Neumann FJ, Schomig A. Tissue factor cytoplasmic domain stimulates migration by activation of the GTPase Rac1 and the mitogen-activated protein kinase p38. *Circulation*. 2005;111:349–355.
69. Annex BH, Denning SM, Channon KM, Sketch MH Jr, Stack RS, Morrissey JH, Peters KG. Differential expression of tissue factor protein in directional atherectomy specimens from patients with stable and unstable coronary syndromes. *Circulation*. 1995;91:619–622.
70. Maier W, Altwegg LA, Corti R, Gay S, Hersberger M, Maly FE, Sutsch G, Roffi M, Neidhart M, Eberli FR, Tanner FC, Gobbi S, von Eckardstein A, Luscher TF. Inflammatory markers at the site of ruptured plaque in acute myocardial infarction: locally increased interleukin-6 and serum amyloid a but decreased C-reactive protein. *Circulation*. 2005;111:1355–1361.
71. Suefuji H, Ogawa H, Yasue H, Kaikita K, Soejima H, Motoyama T, Mizuno Y, Oshima S, Saito T, Tsuji I, Kumeda K, Kamikubo Y, Nakamura S. Increased plasma tissue factor levels in acute myocardial infarction. *Am Heart J*. 1997;134:253–259.
72. Soejima H, Ogawa H, Yasue H, Kaikita K, Nishiyama K, Misumi K, Takazoe K, Miyao Y, Yoshimura M, Kugiyama K, Nakamura S, Tsuji I, Kumeda K. Heightened tissue factor associated with tissue factor pathway inhibitor and prognosis in patients with unstable angina. *Circulation*. 1999;99:2908–2913.
73. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol*. 2000;20:1262–1275.
74. Malarstig A, Tenno T, Johnston N, Lagerqvist B, Axelsson T, Syvanen AC, Wallentin L, Siegbahn A. Genetic variations in the tissue factor gene are associated with clinical outcome in acute coronary syndrome and expression levels in human monocytes. *Arterioscler Thromb Vasc Biol*. 2005;25:2667–2672.

75. Ott I, Koch W, von Beckerath N, de Waha R, Malawaniec A, Mehilli J, Schomig A, Kastrati A. Tissue factor promotor polymorphism-603 A/G is associated with myocardial infarction. *Atherosclerosis*. 2004;177: 189–191.
76. Graham DJ, Campen D, Hui R, Spence M, Cheetham C, Levy G, Shoor S, Ray WA. Risk of acute myocardial infarction and sudden cardiac death in patients treated with cyclo-oxygenase 2 selective and non-selective non-steroidal anti-inflammatory drugs: nested case-control study. *Lancet*. 2005;365:475–481.
77. Molero L, Lopez-Farre A, Mateos-Caceres PJ, Fernandez-Sanchez R, Luisa Maestro M, Silva J, Rodriguez E, Macaya C. Effect of clopidogrel on the expression of inflammatory markers in rabbit ischemic coronary artery. *Br J Pharmacol*. 2005;146:419–424.
78. Kopp CW, Steiner S, Nasel C, Seidinger D, Mlekusch I, Lang W, Bartok A, Ahmadi R, Minar E. Abciximab reduces monocyte tissue factor in carotid angioplasty and stenting. *Stroke*. 2003;34:2560–2567.
79. Osnes LT, Foss KB, Joo GB, Okkenhaug C, Westvik AB, Ovstebo R, Kierulf P. Acetylsalicylic acid and sodium salicylate inhibit LPS-induced NF-kappa B/c-Rel nuclear translocation, and synthesis of tissue factor (TF) and tumor necrosis factor alfa (TNF-alpha) in human monocytes. *Thromb Haemost*. 1996;76:970–976.
80. Seljeflot I, Hurlen M, Arnesen H. Increased levels of soluble tissue factor during long-term treatment with warfarin in patients after an acute myocardial infarction. *J Thromb Haemost*. 2004;2:726–730.
81. Tutar E, Ozcan M, Kilickap M, Gulec S, Aras O, Pamir G, Oral D, Dandelet L, Key NS. Elevated whole-blood tissue factor procoagulant activity as a marker of restenosis after percutaneous transluminal coronary angioplasty and stent implantation. *Circulation*. 2003;108: 1581–1584.
82. Golino P, Ragni M, Cirillo P, Avvedimento VE, Feliciello A, Esposito N, Scognamiglio A, Trimarco B, Iaccarino G, Condorelli M, Chiariello M, Ambrosio G. Effects of tissue factor induced by oxygen free radicals on coronary flow during reperfusion. *Nat Med*. 1996;2:35–40.
83. Iakovou I, Schmidt T, Bonizzoni E, Ge L, Sangiorgi GM, Stankovic G, Airoldi F, Chieffo A, Montorfano M, Carlino M, Michev I, Corvaja N, Briguori C, Gerckens U, Grube E, Colombo A. Incidence, predictors, and outcome of thrombosis after successful implantation of drug-eluting stents. *JAMA*. 2005;293:2126–2130.
84. Leon C, Alex M, Klocke A, Morgenstern E, Moosbauer C, Eckly A, Spannagl M, Gachet C, Engelmann B. Platelet ADP receptors contribute to the initiation of intravascular coagulation. *Blood*. 2004;103:594–600.
85. Cavusoglu E, Chen I, Rappaport J, Marmur JD. Inhibition of tissue factor gene induction and activity using a hairpin ribozyme. *Circulation*. 2002;105:2282–2287.
86. Stephens AC, Rivers RP. Suppression of human monocyte tissue factor synthesis by antisense oligodeoxynucleotide. *Thromb Res*. 1997;85: 387–398.
87. Pendurthi UR, Williams JT, Rao LV. Inhibition of tissue factor gene activation in cultured endothelial cells by curcumin: suppression of activation of transcription factors Egr-1, AP-1, and NF-kappa B. *Arterioscler Thromb Vasc Biol*. 1997;17:3406–3413.
88. Ragni M, Cirillo P, Pascucci I, Scognamiglio A, D'Andrea D, Eramo N, Ezekowitz MD, Pawashe AB, Chiariello M, Golino P. Monoclonal antibody against tissue factor shortens tissue plasminogen activator lysis time and prevents reocclusion in a rabbit model of carotid artery thrombosis. *Circulation*. 1996;93:1913–1918.
89. Erlich JH, Boyle EM, Labriola J, Kovacich JC, Santucci RA, Fearns C, Morgan EN, Yun W, Luther T, Kojikawa O, Martin TR, Pohlman TH, Verrier ED, Mackman N. Inhibition of the tissue factor-thrombin pathway limits infarct size after myocardial ischemia-reperfusion injury by reducing inflammation. *Am J Pathol*. 2000;157:1849–1862.
90. Badimon JJ, Lettino M, Toschi V, Fuster V, Berrozpe M, Chesebro JH, Badimon L. Local inhibition of tissue factor reduces the thrombogenicity of disrupted human atherosclerotic plaques: effects of tissue factor pathway inhibitor on plaque thrombogenicity under flow conditions. *Circulation*. 1999;99:1780–1787.
91. Morrow DA, Murphy SA, McCabe CH, Mackman N, Wong HC, Antman EM. Potent inhibition of thrombin with a monoclonal antibody against tissue factor (Sunol-cH36): results of the PROXIMATE-TIMI 27 trial. *Eur Heart J*. 2005;26:682–688.
92. Kelley RF, Refino CJ, O'Connell MP, Modi N, Sehl P, Lowe D, Pater C, Bunting S. A soluble tissue factor mutant is a selective anticoagulant and antithrombotic agent. *Blood*. 1997;89:3219–3227.
93. Himber J, Refino CJ, Burcklen L, Roux S, Kirchhofer D. Inhibition of arterial thrombosis by a soluble tissue factor mutant and active site-blocked factors IXa and Xa in the guinea pig. *Thromb Haemost*. 2001; 85:475–481.
94. Cirillo P, Golino P, Ragni M, D'Andrea D, Calabro P, Corcione N, Vigorito F, Ravera M, Chiariello M. Long-lasting antithrombotic effects of a single dose of human recombinant, active site-blocked factor VII: insights into possible mechanism(s) of action. *J Thromb Haemost*. 2003; 1:992–998.
95. Jang Y, Guzman LA, Lincoff AM, Gottsauner-Wolf M, Forudi F, Hart CE, Courtman DW, Ezban M, Ellis SG, Topol EJ. Influence of blockade at specific levels of the coagulation cascade on restenosis in a rabbit atherosclerotic femoral artery injury model. *Circulation*. 1995;92: 3041–3050.
96. Abendschein DR, Meng YY, Torr-Brown S, Sobel BE. Maintenance of coronary patency after fibrinolysis with tissue factor pathway inhibitor. *Circulation*. 1995;92:944–949.
97. Tanner FC, Boehm M, Akyurek LM, San H, Yang ZY, Tashiro J, Nabel GJ, Nabel EG. Differential effects of the cyclin-dependent kinase inhibitors p27(Kip1), p21(Cip1), and p16(Ink4) on vascular smooth muscle cell proliferation. *Circulation*. 2000;101:2022–2025.
98. Nishida T, Ueno H, Atsuchi N, Kawano R, Asada Y, Nakahara Y, Kamikubo Y, Takeshita A, Yasui H. Adenovirus-mediated local expression of human tissue factor pathway inhibitor eliminates shear stress-induced recurrent thrombosis in the injured carotid artery of the rabbit. *Circ Res*. 1999;84:1446–1452.
99. Zoldhelyi P, Chen ZQ, Shelat HS, McNatt JM, Willerson JT. Local gene transfer of tissue factor pathway inhibitor regulates intimal hyperplasia in atherosclerotic arteries. *Proc Natl Acad Sci U S A*. 2001;98: 4078–4083.
100. Abraham E, Reinhart K, Opal S, Demeyer I, Doig C, Rodriguez AL, Beale R, Svoboda P, Laterre PF, Simon S, Light B, Spapen H, Stone J, Seibert A, Peckelsen C, De Deyne C, Postier R, Pettila V, Artigas A, Percell SR, Shu V, Zwingelstein C, Tobias J, Poole L, Stolzenbach JC, Creasey AA. Efficacy and safety of tifacogin (recombinant tissue factor pathway inhibitor) in severe sepsis: a randomized controlled trial. *JAMA*. 2003;290:238–247.
101. Moons AH, Peters RJ, Bijsterveld NR, Piek JJ, Prins MH, Vlasuk GP, Rote WE, Buller HR. Recombinant nematode anticoagulant protein c2, an inhibitor of the tissue factor/factor VIIa complex, in patients undergoing elective coronary angioplasty. *J Am Coll Cardiol*. 2003;41: 2147–2153.

Special Report

Drug-Eluting Stent and Coronary Thrombosis
Biological Mechanisms and Clinical Implications

Thomas F. Lüscher, MD; Jan Steffel, MD; Franz R. Eberli, MD; Michael Joner, MD; Gaku Nakazawa, MD; Felix C. Tanner, MD; Renu Virmani, MD

Abstract—Although rare, stent thrombosis remains a severe complication after stent implantation owing to its high morbidity and mortality. Since the introduction of drug-eluting stents (DES), most interventional centers have noted stent thrombosis up to 3 years after implantation, a complication rarely seen with bare-metal stents. Some data from large registries and meta-analyses of randomized trials indicate a higher risk for DES thrombosis, whereas others suggest an absence of such a risk. Several factors are associated with an increased risk of stent thrombosis, including the procedure itself (stent malapposition and/or underexpansion, number of implanted stents, stent length, persistent slow coronary blood flow, and dissections), patient and lesion characteristics, stent design, and premature cessation of antiplatelet drugs. Drugs released from DES exert distinct biological effects, such as activation of signal transduction pathways and inhibition of cell proliferation. As a result, although primarily aimed at preventing vascular smooth muscle cell proliferation and migration (ie, key factors in the development of restenosis), they also impair reendothelialization, which leads to delayed arterial healing, and induce tissue factor expression, which results in a prothrombogenic environment. In the same way, polymers used to load these drugs have been associated with DES thrombosis. Finally, DES impair endothelial function of the coronary artery distal to the stent, which potentially promotes the risk of ischemia and coronary occlusion. Although several reports raise the possibility of a substantially higher risk of stent thrombosis in DES, evidence remains inconclusive; as a consequence, both large-scale and long-term clinical trials, as well as further mechanistic studies, are needed. The present review focuses on the pathophysiological mechanisms and pathological findings of stent thrombosis in DES. (***Circulation*. 2007;115:1051-1058.**)

Key Words: stents ■ thrombosis ■ pathology ■ physiology ■ risk factors ■ arteries ■ myocardial infarction

With the introduction of balloon-expandable stents, coronary remodeling and, in turn, restenosis were reduced compared with angioplasty alone.[1,2] With the risk of restenosis still in the range of 15% to 20%, however, drug-eluting stents (DES) designed to release pharmacological agents after deployment were developed to inhibit the response to injury mainly responsible for restenosis after bare-metal stent (BMS) implantation (ie, vascular smooth muscle cell migration and proliferation and proteoglycan deposition). As a result, restenosis and target-vessel revascularization could be reduced to rates below 10% after DES implantation.[3,4]

In the first series of patients receiving BMS, stent thrombosis was already recognized as a severe complication after implantation owing to its high mortality. With the introduction of P2Y-receptor antagonists (ie, ticlopidine, clopidogrel) for platelet inhibition in combination with acetylsalicylic acid, the incidence of stent thrombosis decreased substantially in stable patients to levels as low as 1%.[5] Most of the events occurred within the first 10 days after implantation; indeed, stent thrombosis after the first month was extremely rare with BMS.[6,7]

Despite reduced restenosis rates, the frequency of in-stent thrombosis has not decreased with DES compared with BMS.[8–12] Indeed, several hundred cases of stent thrombosis have been reported for rapamycin-coated stents.[13] A number of reports imply that thrombosis rates of DES may even be higher in the "real world" than in clinical trials.[14,15] Notably, many operators have experienced very late stent thrombosis (3 years after implantation and beyond) in a number of patients, which was not seen with BMS. This review will briefly discuss presently available clinical data and focus on pathophysiological mechanisms of in-stent thrombosis in DES.

Clinical Evidence

Numerous reports describe the occurrence of acute (<24 hours), subacute (<30 days), late (>30 days), and very late (>12 months) stent thrombosis after DES implantation.[8,13,16] However, the true incidence of stent thrombosis may be underestimated in clinical trials and could occur at substantially higher rates in the "real-world" setting, where more

From Cardiology (T.F.L., J.S., F.R.E., F.C.T.), Cardiovascular Center, University Hospital Zürich, Zürich, Switzerland; Cardiovascular Research (T.F.L., J.S., F.C.T.), Physiology Institute, and Center for Integrative Human Physiology (T.F.L., J.S., F.C.T.), University of Zürich, Zürich, Switzerland; and CVPath Institute, Inc (M.J., G.N., R.V.), International Registry of Pathology, Gaithersburg, Md.

Correspondence to Thomas F. Lüscher, MD, Department of Cardiology, University of Zurich, Rämistrasse 100, 8091 Zürich, Switzerland. E-mail cardiotfl@gmx.ch

© 2007 American Heart Association, Inc.

Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/CIRCULATIONAHA.106.675934**

complex lesions are treated.[14,15] Given the small size of both clinical trials and registries and the low absolute number of stent thromboses, meta-analyses were performed to clarify this issue. Several meta-analyses initially did not reveal an increased risk of stent thrombosis with DES compared with BMS at 9 to 12 months.[10,11,12] Subsequently, 3 additional meta-analyses attracted special attention: In a large analysis of >8000 patients from 2 academic referral hospitals, a substantial cumulative incidence of angiographically documented DES thrombosis was noted (2.9%), yielding a rate of 1.3 per 100 patient-years.[17] In that analysis, however, no patients with BMS were included, and hence, direct comparison of the 2 types of stents was not possible; given the change in percutaneous coronary intervention practice with DES, these data are difficult to interpret. In another study directly comparing DES and BMS, an increase in late thrombosis was noted in DES.[18] Furthermore, a meta-analysis that included total mortality and Q-wave myocardial infarction as end points representing the "inclusive clinical surrogate of stent thrombosis" found an increased cumulative incidence of death or myocardial infarction at the latest available follow-up.[9] Because coronary patients experience new occlusions at sites distant from the implanted stent and/or fatal arrhythmias, however, such an analysis may overestimate the true stent thrombosis rate. Moreover, interpretation of meta-analyses is restricted because of their inherent limitations, which include selective use of end points, incomplete data sets, and the retrospective nature of their analysis.

Pathophysiology of In-Stent Thrombosis

Several factors that contribute to stent thrombosis have been recognized, which are discussed below.

Procedure-Related Factors

Among the procedure-related factors, smaller final lumen dimensions (stent malapposition and/or underexpansion), stent length, persistent slow coronary blood flow, placement of multiple stents, positive remodeling, dissections, geographic miss, and late stent malapposition due to thrombus resolution appear to be most important for the development of in-stent thrombosis.[19–25] Also, in DES, stent length, stent underexpansion, and residual stenosis have been observed to correlate with an increased risk for stent thrombosis.[26,27] These factors are of great interest because they can be avoided during the intervention, but they are unlikely to differ between BMS and DES.

Patient- and Lesion-Related Factors

Several patient-related factors have been associated with the development of in-stent thrombosis, including low ejection fraction,[21] diabetes mellitus,[28] advanced age,[22] and stenting in the setting of an acute coronary syndrome.[26] Similarly, in DES, primary stenting in acute myocardial infarction, diabetes mellitus, renal failure, and low ejection fraction appear to be associated with an increased risk for stent thrombosis.[14,26,29,30] In particular, the increased risk in patients with acute coronary syndrome could be due to delayed healing, lack of endothelialization, and presence of a pronounced inflammatory and thrombogenic environment of the exposed necrotic core to flowing blood, accompanied by enhanced platelet reactivity; furthermore, rapamycin and paclitaxel potentiate thrombin-induced expression of tissue factor (see below).

Furthermore, certain lesion characteristics are reported to be associated with an increased risk of stent thrombosis. In DES, this pertains in particular to stenting of bifurcation lesions or in-stent restenosis lesions.[14,29,30] In addition, interventional practice has changed, with advocates of "normal to normal" coronary artery stenting and revascularization of more complex lesions that carry a higher risk of stent thrombosis. This latter aspect makes comparisons of registries with historical controls difficult.

Antiplatelet Therapy

Stents are foreign bodies in the vessel wall and thus induce platelet adhesion and activation of the coagulation cascade. Furthermore, high-pressure implantation with noncompliant balloons induces significant vascular injury, with exposure of thrombogenic molecules of the subintima and media (including plaque material) to the blood stream. As a consequence, only potent platelet inhibition made the procedure feasible, and antiplatelet hyporesponsiveness has been associated with an increased risk for stent thrombosis.[31] In line with this observation, discontinuation of antiplatelet therapy has been observed to be particularly associated with DES thrombosis.[8,16,32] The appropriate duration of the long-term antiplatelet regimen for prevention of DES thrombosis remains to be assessed in randomized prospective trials; at present, a course of 12 months of dual-antiplatelet therapy may be considered especially in high-risk, real-world patients.

Thrombogenicity of the Stent

A predisposition for the development of stent thrombosis has been observed with certain stent materials; for example, platelet activation was greater during the 30 days after implantation of an open-cell versus a closed-cell stent.[33] Stent strut thickness and polymer type and thickness also play an important role. It has been reported previously that the nonerodable polymers of the Cypher and Taxus DES provoke chronic eosinophilic infiltration of the arterial wall, suggestive of hypersensitivity reactions in a small number of cases.[7,34] However, the causal relationship between polymer-induced inflammation and the incidence of late stent thrombosis has only been proven in a minority of patients possessing a proinflammatory phenotype. Detailed analysis of the morphological changes shows a localized immune response, with predominance of CD45-positive lymphocytes and eosinophils. In fact, our experience shows that all cases of hypersensitivity occur >4 months after DES implantation.[7] The preclinical experience in a pig model also shows a progressive increase in the presence of granulomatous reactions, including eosinophilic infiltrate, starting at 28 days after Cypher stent implantation: 1 month, 14%; 3 months, 43%; 6 months, 60% (R.V., unpublished data). One possible explanation of these findings is that the hypersensitivity reaction peaks after the complete release of the drug and is likely related to the polymer. In addition, positive remodeling

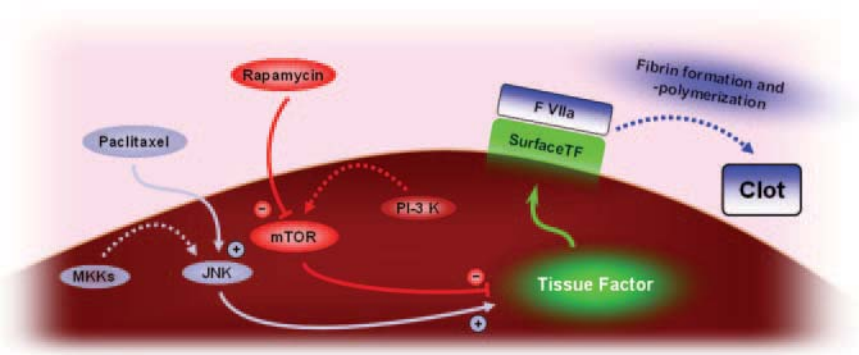


Figure 1. Rapamycin and paclitaxel increase tissue factor (TF) expression. Paclitaxel enhances c-Jun NH_2-terminal kinase (JNK) phosphorylation, which in turn leads to an increase in TF protein expression and TF surface activity. The PI3-kinase and its downstream target, the mammalian target of rapamycin (mTOR), inhibit endothelial TF expression; rapamycin inhibits the mammalian target of rapamycin, which leads to a disinhibition of (and thus an increase in) TF expression and surface activity. MKKs indicates map kinase kinases (upstream regulators of JNK); PI-3K, phosphatidylinositol-3 kinase.

has been observed in vessels showing a hypersensitivity reaction.

Furthermore, drugs loaded on DES may exert a prothrombogenic effect. Rapamycin (sirolimus), a macrocyclic lactone, is used on DES, because it is known to inhibit proliferation and migration of vascular smooth muscle cells, important factors in the development of neointima formation and restenosis, through interference with cell cycle regulators.[35,36] On a subcellular level, rapamycin binds to the FK-binding protein 12 and subsequently inhibits the mammalian target of rapamycin. The mammalian target of rapamycin is a downstream target of the phosphatidylinositol-3 kinase pathway, which in turn is involved in an inhibitory fashion in the regulation of tissue factor in endothelial cells and monocytes.[37–39] As a result, rapamycin inhibition of the mammalian target of rapamycin increases both thrombin- and tumor necrosis factor-α–induced endothelial tissue factor expression and activity at concentrations of rapamycin that are encountered in vivo (Figure 1).[38,40]

Paclitaxel is a lipophilic diterpenoid that binds to the β-subunit of the tubulin heterodimer, promoting tubulin polymerization, cell cycle arrest, and, eventually, inhibition of vascular smooth muscle cell migration and proliferation.[41,42] In addition, paclitaxel is known to activate c-Jun NH_2-terminal kinase,[43,44] an important mediator of endothelial and monocytic tissue factor induction.[37,39,45] Consequently, paclitaxel also enhances tissue factor expression and activity in endothelial cells[44]; again, concentrations used in this in vitro study are comparable with local tissue concentrations of paclitaxel after stent deployment.[46]

In sirolimus-eluting stents, ≈80% of the rapamycin has eluted by 30 days, whereas paclitaxel-eluting stents have a biphasic drug release profile in vitro with an initial burst during the first 48 hours after implantation followed by a sustained low-level release for at least 2 weeks.[47] However, both rapamycin and paclitaxel easily penetrate into cells of the vessel wall owing to their lipophilic properties, which leads to chronic retention of the drug in the arterial tissue.[19,46,48] Thus, both rapamycin- and paclitaxel-induced tissue factor expression may contribute to a prothrombotic environment after deployment of DES, particularly in the acute and subacute setting and possibly in late stent thrombosis (Figure 1). The relationship between these findings and stent thrombosis in clinical practice requires further study, particularly to examine the degree and the spatiotemporal pattern of tissue factor expression in the arterial wall after deployment of DES.

Impaired Reendothelialization

Reendothelialization occurs after vascular injury and similarly after stent placement. Traditionally, it was believed that endothelial cells proliferate and migrate from intact neighboring coronary segments, eventually leading to the reendothelialization of the injured segment. In vitro, rapamycin and paclitaxel not only inhibit proliferation and migration of vascular smooth muscle cells but equally suppress endothelial cells,[7,35,36,38,49–51] thereby potentially impeding reendothelialization (Figure 2). We observed poor endothelial cell junction formation and microthrombi of focal platelet aggregation at 16 months after rapamycin stent implantation in a patient dying of a non-DES–related cause.[52] It has been proposed that bone marrow–derived endothelial progenitor cells may also be involved in reendothelialization.[53,54] Interestingly, rapamycin inhibits proliferation, migration, and differentiation of human endothelial progenitor cells in vitro.[55,56] Hence, drugs loaded on DES may affect the number as well as the homing and proliferation of endothelial progenitor cells, thus further preventing proper endothelial healing (Figure 2).

In vivo, the time course of endothelial healing after stent implantation varies in different animal models. Although in healthy pigs, endothelialization is similar between BMS and DES at 28 days,[48] a clear delay of endothelialization in both sirolimus- and paclitaxel-eluting stents was observed in a rabbit iliac-overlapping DES implantation model.[46] In view of distinct differences with respect to vessel wall reactivity after stent implantation between animals and humans, however, these results cannot be applied entirely to the situation in humans. After BMS implantation, near-complete endothelialization has been suggested to occur by 3 to 4 months.[6] A morphological autopsy study comparing coronary segments from patients after DES and BMS implantation revealed delayed arterial healing and poorer endothelialization after DES compared with BMS implantation of similar duration (Figure 3).[7] Indeed, in 23 DES patients in that study, 14 had evidence of late-stent thrombosis, and of these 14 patients, 13 died of a DES-related cause.[7] Thus, current evidence suggests delayed reendothelialization and arterial healing after implantation of DES compared with BMS, resulting in potentially

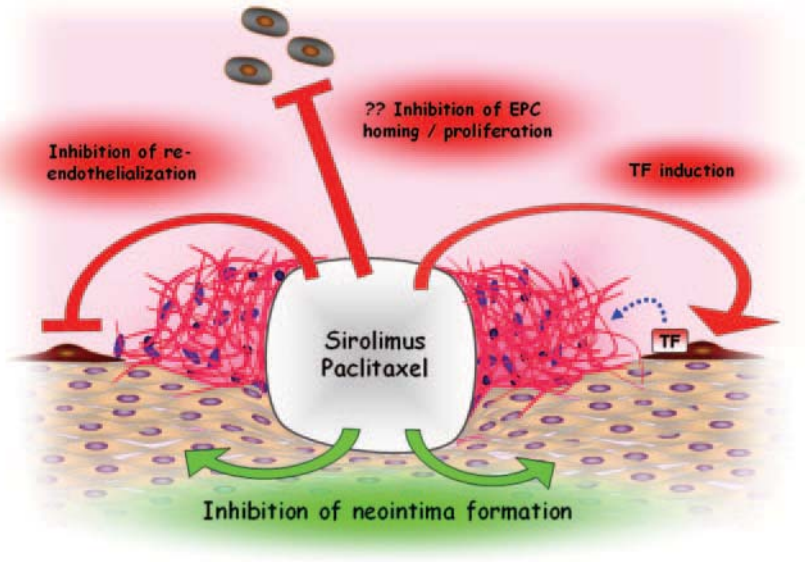


Figure 2. DES reduce neointima formation but may increase stent thrombogenicity. Effect of sirolimus-eluting/paclitaxel-eluting stent strut on the local vessel wall after implantation. Sirolimus/paclitaxel reduces neointima formation by inhibiting vascular smooth muscle migration and proliferation (green arrows). However, the drugs also inhibit reendothelialization, induce tissue factor (TF), and may prevent homing and proliferation of endothelial progenitor cells (EPCs; red arrows/bars).

enhanced thrombogenicity (Figure 4). It is uncertain whether reendothelialization with DES is only delayed or persistently incomplete up to late time points. It is unclear in atherosclerotic human arteries how long it will take for DES to endothelialize. We have shown that in humans, delayed healing is common with current DES and that in those that thrombose, other factors, such as hypersensitivity reaction, bifurcating and ostial stenting, penetration of

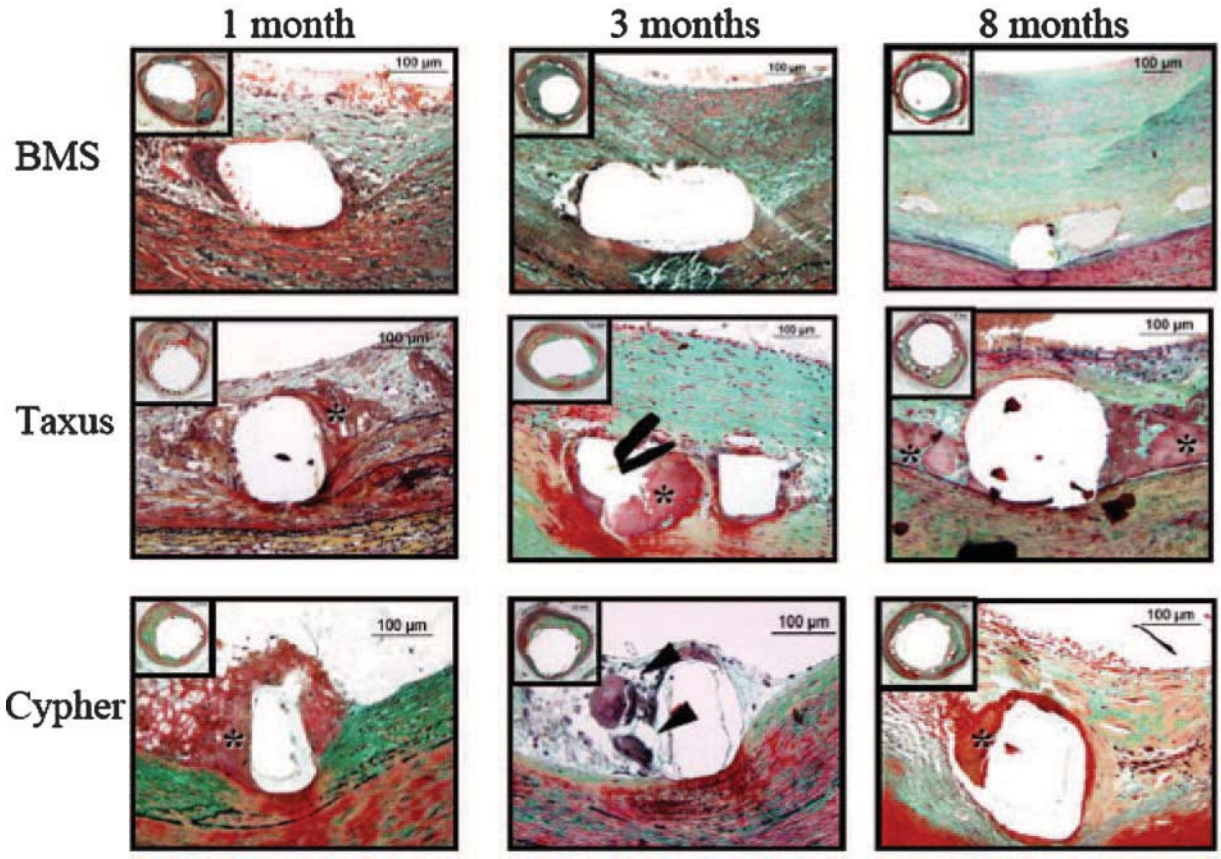


Figure 3. Delayed reendothelialization after DES implantation. Time course of arterial healing in BMS, Taxus DES, and Cypher DES from 1 to 8 months after stent implantation. Although some peristrut inflammation is observed in BMS at 1 month, complete arterial healing, including a well-established neointimal layer, is seen at 3 and 8 months' duration. Taxus DES shows early fibrin deposition surrounding stent struts (*), which persists up to 8 months, as a sign of delayed healing. In contrast, Cypher DES shows predominance of inflammatory cells, including giant cell formation (black arrowheads), at early time points (1 and 3 months), whereas fibrin deposition is stronger at 8 months.

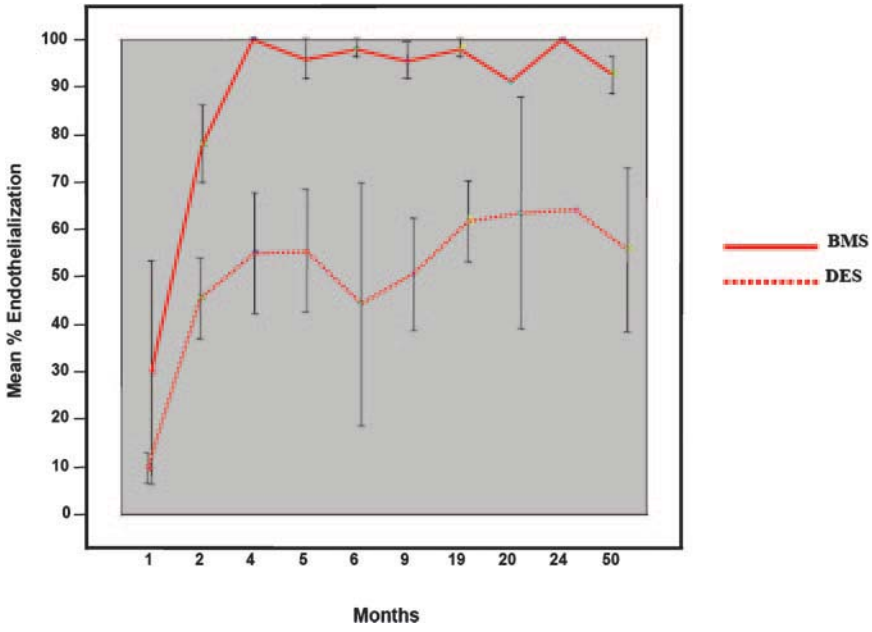


Figure 4. Temporal sequence of reendothelialization in BMS and DES.

a necrotic core, stent malapposition, and restenosis, may also be important predictors of thrombosis.

There is compelling evidence that certain subsets of patients have a favorable long-term outcome after implantation of DES, which results in a reduced need for interventional or surgical revascularization due to sustained suppression of neointimal growth. Factors associated with greater healing from our experience include shorter stent length, less plaque area, less fibrin deposition, and greater endothelialization. Other factors that may influence healing are likely to be patient-related, such as antiplatelet therapy discontinuation, renal failure, diabetes mellitus, and a lower ejection fraction, which have all been reported in clinical studies.[14] Furthermore, there is variability from patient to patient even in wound healing; the response to drug also varies, with some patients requiring a lower drug dose for equivalent benefit. Thus, many factors influence the healing process and vary with individual risk factors.

Risk Factors for Different Time Points of DES Thrombosis

Stent thrombosis may occur acutely (within 24 hours of stent placement), subacutely (up to 30 days after stent implantation), as late thrombosis (after 30 days), or as very late thrombosis (after 12 months). The most important risk factors for acute and subacute stent thrombosis are primary stenting in ST-segment elevation myocardial infarction and acute coronary syndromes.[20,26] Additional risk factors include stent length, congestive heart failure, and a prothrombogenic state, such as metastatic cancer.[19,20,26,27] One of the most significant risk factors for late and very late stent thrombosis appears to be discontinuation of antiplatelet therapy.[26,27] Other predictors are stent underexpansion and residual reference segment stenosis.[19,27]

Design of Future DES

The stent coating influences thrombogenicity. Whether a simple chemical coating, such as titanium-nitride-oxide, that diminishes platelet adhesion and fibrinogen binding compared with stainless steel will be effective against restenosis and stent thrombosis remains to be elucidated in large clinical trials.[57] Coating of stents with substances that potentially facilitate reendothelialization may represent a novel therapeutic approach. For example, in preliminary studies, coating of stents with CD34 antibodies designed to "capture" endothelial progenitor cells proved to accelerate endothelial coverage and appeared safe and feasible in humans.[58] Similarly, stents loaded with an integrin-binding cyclic Arg-Gly-Asp peptide accelerated endothelialization by attracting endothelial progenitor cells in a porcine model.[59] Further studies are needed to assess the long-term efficacy and safety of these biologically active stents. Furthermore, a combination of "prohealing" substances (such as vascular endothelial growth factor) with established "antirestenosis" drugs may represent an interesting approach to obtain the benefit of reduced restenosis without the cost of an increased risk for stent thrombosis.

Considering the delay in healing along with the unknown time of reendothelialization of current DES, prohealing strategies such as the use of peroxisome proliferator–activated receptor-γ agonists, which not only diminish inflammation but also enhance endothelialization, may also represent an interesting new approach for DES.[60] Given the importance of tissue factor in the initiation of coagulation and thrombosis, we also proposed dimethyl sulfoxide as a novel coating strategy for DES.[61] Dimethyl sulfoxide prevents vascular smooth muscle cell proliferation and migration, ie, the key mechanisms of restenosis; at the same time, dimethyl sulfoxide inhibits tissue factor upregulation in endothelial cells, vascular smooth muscle, and monocytes and prevents thrombotic occlusion in a mouse carotid injury model.[61]

Conclusions

The pathogenesis of stent thrombosis is still not fully understood. A combination of factors may be involved, including procedure-related factors, patient-related factors, and lesion

characteristics. With DES, biological properties such as thrombogenicity, ie, induction of tissue factor, inhibition of reendothelialization of the stented segment, and distal endothelial dysfunction may increase the risk beyond that seen with BMS. Because premature cessation and resistance to antiplatelet drugs is associated with subacute and late DES thrombosis, prolonged dual-platelet inhibition must be considered, especially in high-risk patients, balanced against the risk of bleeding.

With nearly 6 million DES implanted, and in view of the high morbidity and mortality associated with it, stent thrombosis is an important healthcare issue that requires further clinical study. Consistent with this interpretation, the US Food and Drug Administration issued a statement acknowledging the importance of the increasing concern with respect to DES thrombosis. There was consensus that the on-label use of DES may be associated with a small increase in stent thrombosis compared with bare-metal stents, but that it does not lead to an increased risk of death or myocardial infarction, and that under these conditions, the benefits of DES outweigh a possibly increased risk for stent thrombosis[62]; the panel left open the possibility, however, that off-label use of DES (ie, use of DES outside the FDA-approved indications) may indeed be associated with an increased risk of death or myocardial infarction compared with the use of BMS owing to an increase in the rate of stent thrombosis.[62]

Statements that second-generation DES appear to be less prone to stent thrombosis and thus "safer" than first-generation DES appear equally premature because the same uncertainty with respect to long-term safety prevails. Additional in vitro studies of compounds used on DES and autopsy studies of patients with DES dying of cardiac and noncardiac causes would help in understanding the mechanisms of stent thrombosis. Moreover, large-scale, non–corporate-sponsored registries and clinical trials are needed to reliably assess the "true" risk of stent thrombosis with DES. Control of data by non–corporate-sponsored data analysis centers under the auspices of the American Heart Association/American College of Cardiology/European Society of Cardiology, including cardiac surgeons, cardiologists, internists, and cardiac pathologists, is a prerequisite to the unbiased evaluation of current and future DES technologies.

Sources of Funding

Research of the authors was supported by the Swiss National Research Foundation (grant No. 3100-068118.02/1 to Dr Lüscher and 3200B0-113328/1 to Dr Tanner) and by the European Union (grant No. G5RD-CT-2001-00532 to Dr Lüscher) and the Bonizzi-Theler Stiftung (to Dr Steffel and Dr Tanner). The authors received unrestricted research grants from Cordis, Biotronik, Boston Scientific, and Medtronic (Dr Lüscher and Dr Eberli). Dr Virmani has received research grants from companies including Medtronic, Abbott, Guidant, and Cordis.

Disclosures

Dr Lüscher and Dr Tanner hold a patent on the potential clinical applications of dimethyl sulfoxide. Dr Virmani is a consultant to Medtronic and Guidant. The remaining authors report no conflicts.

References

1. Serruys PW, de Jaegere P, Kiemeneij F, Macaya C, Rutsch W, Heyndrickx G, Emanuelsson H, Marco J, Legrand V, Materne P, Belardi J, Sigwart U, Colombo A, Goy JJ, van den Heuvel P, Delcan J, Morel M-A; Benestent Study Group. A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. *N Engl J Med.* 1994;331:489–495.
2. Fischman DL, Leon MB, Baim DS, Schatz RA, Savage MP, Penn I, Detre K, Veltri L, Ricci D, Nobuyoshi M, Cleman M, Heuser R, Almond D, Teirstein PS, Fish RD, Colombo A, Brinker J, Moses J, Shaknovich A, Hirshfeld J, Bailey S, Ellis S, Rake R, Goldberg S; Stent Restenosis Study Investigators. A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. *N Engl J Med.* 1994;331:496–501.
3. Morice MC, Serruys PW, Sousa JE, Fajadet J, Ban Hayashi E, Perin M, Colombo A, Schuler G, Barragan P, Guagliumi G, Molnar F, Falotico R. A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. *N Engl J Med*. 2002;346:1773–1780.
4. Moses JW, Leon MB, Popma JJ, Fitzgerald PJ, Holmes DR, O'Shaughnessy C, Caputo RP, Kereiakes DJ, Williams DO, Teirstein PS, Jaeger JL, Kuntz RE. Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery. *N Engl J Med*. 2003; 349:1315–1323.
5. Bertrand ME, Rupprecht HJ, Urban P, Gershlick AH. Double-blind study of the safety of clopidogrel with and without a loading dose in combination with aspirin compared with ticlopidine in combination with aspirin after coronary stenting: the Clopidogrel Aspirin Stent International Cooperative Study (CLASSICS). *Circulation*. 2000;102:624–629.
6. Farb A, Burke AP, Kolodgie FD, Virmani R. Pathological mechanisms of fatal late coronary stent thrombosis in humans. *Circulation*. 2003;108: 1701–1706.
7. Joner M, Finn AV, Farb A, Mont EK, Kolodgie FD, Ladich E, Kutys R, Skorija K, Gold HK, Virmani R. Pathology of drug-eluting stents in humans: delayed healing and late thrombotic risk. *J Am Coll Cardiol*. 2006;48:193–202.
8. McFadden EP, Stabile E, Regar E, Cheneau E, Ong AT, Kinnaird T, Suddath WO, Weissman NJ, Torguson R, Kent KM, Pichard AD, Satler LF, Waksman R, Serruys PW. Late thrombosis in drug-eluting coronary stents after discontinuation of antiplatelet therapy. *Lancet*. 2004;364: 1519–1521.
9. Camenzind E, Steg PG, Wijns W. Safety of drug-eluting stents: a meta-analysis of 1st generation DES programs. Abstract presented at: World Congress of Cardiology 2006, September 2–6, 2006, Barcelona, Spain.
10. Moreno R, Fernandez C, Hernandez R, Alfonso F, Angiolillo DJ, Sabate M, Escaned J, Banuelos C, Fernandez-Ortiz A, Macaya C. Drug-eluting stent thrombosis: results from a pooled analysis including 10 randomized studies. *J Am Coll Cardiol*. 2005;45:954–959.
11. Bavry AA, Kumbhani DJ, Helton TJ, Bhatt DL. What is the risk of stent thrombosis associated with the use of paclitaxel-eluting stents for percutaneous coronary intervention? A meta-analysis. *J Am Coll Cardiol*. 2005;45:941–946.
12. Bavry AA, Kumbhani DJ, Helton TJ, Bhatt DL. Risk of thrombosis with the use of sirolimus-eluting stents for percutaneous coronary intervention (from registry and clinical trial data). *Am J Cardiol*. 2005;95:1469–1472.
13. FDA advises physicians of adverse events associated with Cordis Cypher coronary stents. US Food and Drug Administration Public Health Web Notification. October 29, 2003;T03–T71. Available at: http://www.fda.gov/bbs/topics/ANSWERS/2003/ANS01257.html. Accessed February 10, 2007.
14. Iakovou I, Schmidt T, Bonizzoni E, Ge L, Sangiorgi GM, Stankovic G, Airoldi F, Chieffo A, Montorfano M, Carlino M, Michev I, Corvaja N, Briguori C, Gerckens U, Grube E, Colombo A. Incidence, predictors, and outcome of thrombosis after successful implantation of drug-eluting stents. *JAMA*. 2005;293:2126–2130.
15. Ong AT, McFadden EP, Regar E, de Jaegere PP, van Domburg RT, Serruys PW. Late angiographic stent thrombosis (LAST) events with drug-eluting stents. *J Am Coll Cardiol*. 2005;45:2088–2092.
16. Pfisterer ME, Kaiser CA, Bader F, Brunner-La Rocca HP, Bonetti PO, Buser PT Late clinical events related to late stent thrombosis after stopping clopidogrel: prospective randomized comparison between drug-eluting versus bare-metal stenting. In: Program and abstracts from the American College of Cardiology 55th Annual Scientific Session; March 11–14, 2006; Atlanta, Ga. Abstract 422-11.
17. Daemen J, Wenaweser P, Tsuchida K, Vaina S, Abrecht L, Morger C, Kukreja N, Jüni P, Sianos G, Hellige G, van Domburg R, Hess OM,

Boersma E, Meier B, Windecker S, Serruys P. Early and late coronary stent thrombosis of sirolimus-eluting and paclitaxel-eluting stents in routine clinical practice: data from a large two-institutional cohort study. *Lancet*. In press.
18. Bavry AA, Kumbhani DJ, Helton TJ, Borek PP, Mood GR, Bhatt DL. Late thrombosis of drug-eluting stents: a meta-analysis of randomized clinical trials. *Am J Med*. 2006;119:1056–1061.
19. Kereiakes DJ, Choo JK, Young JJ, Broderick TM. Thrombosis and drug-eluting stents: a critical appraisal. *Rev Cardiovasc Med*. 2004; 5:9–15.
20. Orford JL, Lennon R, Melby S, Fasseas P, Bell MR, Rihal CS, Holmes DR, Berger PB. Frequency and correlates of coronary stent thrombosis in the modern era: analysis of a single center registry. *J Am Coll Cardiol*. 2002;40:1567–1572.
21. Moussa I, Di Mario C, Reimers B, Akiyama T, Tobis J, Colombo A. Subacute stent thrombosis in the era of intravascular ultrasound-guided coronary stenting without anticoagulation: frequency, predictors and clinical outcome. *J Am Coll Cardiol*. 1997;29:6–12.
22. Schuhlen H, Kastrati A, Dirschinger J, Hausleiter J, Elezi S, Wehinger A, Pache J, Hadamitzky M, Schomig A. Intracoronary stenting and risk for major adverse cardiac events during the first month. *Circulation*. 1998; 98:104–111.
23. Cheneau E, Leborgne L, Mintz GS, Kotani J, Pichard AD, Satler LF, Canos D, Castagna M, Weissman NJ, Waksman R. Predictors of subacute stent thrombosis: results of a systematic intravascular ultrasound study. *Circulation*. 2003;108:43–47.
24. Cutlip DE, Baim DS, Ho KK, Popma JJ, Lansky AJ, Cohen DJ, Carrozza JP Jr, Chauhan MS, Rodriguez O, Kuntz RE. Stent thrombosis in the modern era: a pooled analysis of multicenter coronary stent clinical trials. *Circulation*. 2001;103:1967–1971.
25. Chieffo A, Bonizzoni E, Orlic D, Stankovic G, Rogacka R, Airoldi F, Mikhail GW, Montorfano M, Michev I, Carlino M, Colombo A. Intraprocedural stent thrombosis during implantation of sirolimus-eluting stents. *Circulation*. 2004;109:2732–2736.
26. Park DW, Park SW, Park KH, Lee BK, Kim YH, Lee CW, Hong MK, Kim JJ, Park SJ. Frequency of and risk factors for stent thrombosis after drug-eluting stent implantation during long-term follow-up. *Am J Cardiol*. 2006;98:352–356.
27. Fujii K, Carlier SG, Mintz GS, Yang YM, Moussa I, Weisz G, Dangas G, Mehran R, Lansky AJ, Kreps EM, Collins M, Stone GW, Moses JW, Leon MB. Stent underexpansion and residual reference segment stenosis are related to stent thrombosis after sirolimus-eluting stent implantation: an intravascular ultrasound study. *J Am Coll Cardiol*. 2005;45:995–998.
28. Silva JA, Ramee SR, White CJ, Collins TJ, Jenkins JS, Nunez E, Zhang S, Jain SP. Primary stenting in acute myocardial infarction: influence of diabetes mellitus in angiographic results and clinical outcome. *Am Heart J*. 1999;138:446–455.
29. Ong AT, Hoye A, Aoki J, van Mieghem CA, Rodriguez Granillo GA, Sonnenschein K, Regar E, McFadden EP, Sianos G, van der Giessen WJ, de Jaegere PP, de Feyter P, van Domburg RT, Serruys PW. Thirty-day incidence and six-month clinical outcome of thrombotic stent occlusion after bare-metal, sirolimus, or paclitaxel stent implantation. *J Am Coll Cardiol*. 2005;45:947–953.
30. Kuchulakanti PK, Chu WW, Torguson R, Ohlmann P, Rha SW, Clavijo LC, Kim SW, Bui A, Gevorkian N, Xue Z, Smith K, Fournadjieva J, Suddath WO, Satler LF, Pichard AD, Kent KM, Waksman R. Correlates and long-term outcomes of angiographically proven stent thrombosis with sirolimus- and paclitaxel-eluting stents. *Circulation*. 2006;113: 1108–1113.
31. Wenaweser P, Dorffler-Melly J, Imboden K, Windecker S, Togni M, Meier B, Haeberli A, Hess OM. Stent thrombosis is associated with an impaired response to antiplatelet therapy. *J Am Coll Cardiol*. 2005;45: 1748–1752.
32. Eisenstein EL, Anstrom KJ, Kong DF, Shaw LK, Tuttle RH, Mark DB, Kramer JM, Harrington RA, Matchar DB, Kandzari DE, Peterson ED, Schulman KA, Califf RM. Clopidogrel use and long-term clinical outcomes after drug-eluting stent implantation. *JAMA*. 2006;297: 159–168.
33. Gurbel PA, Callahan KP, Malinin AI, Serebruany VL, Gillis J. Could stent design affect platelet activation? Results of the Platelet Activation in STenting (PAST) Study. *J Invasive Cardiol*. 2002;14:584–589.
34. Nebeker JR, Virmani R, Bennett CL, Hoffman JM, Samore MH, Alvarez J, Davidson CJ, McKoy JM, Raisch DW, Whisenant BK, Yarnold PR, Belknap SM, West DP, Gage JE, Morse RE, Gligoric G, Davidson L, Feldman MD. Hypersensitivity cases associated with drug-eluting coronary stents: a review of available cases from the Research on Adverse Drug Events and Reports (RADAR) project. *J Am Coll Cardiol*. 2006; 47:175–181.
35. Marx SO, Jayaraman T, Go LO, Marks AR. Rapamycin-FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. *Circ Res*. 1995;76:412–417.
36. Poon M, Marx SO, Gallo R, Badimon JJ, Taubman MB, Marks AR. Rapamycin inhibits vascular smooth muscle cell migration. *J Clin Invest*. 1996;98:2277–2283.
37. Steffel J, Luscher TF, Tanner FC. Tissue factor in cardiovascular diseases: molecular mechanisms and clinical implications. *Circulation*. 2006;113:722–731.
38. Steffel J, Latini RA, Akhmedov A, Zimmermann D, Zimmerling P, Luscher TF, Tanner FC. Rapamycin, but not FK-506, increases endothelial tissue factor expression: implications for drug-eluting stent design. *Circulation*. 2005;112:2002–2011.
39. Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J Biol Chem*. 2002;277:32124–32132.
40. US Food and Drug Administration. Center for Devices and Radiological Health. Cypher Sirolimus-eluting coronary stent on RAPTOR over-the-wire delivery system or RAPTORRAIL rapid exchange delivery system. Available at: http://www.fda.gov/cdrh/pdf2/p020026.html. Accessed December 17, 2004.
41. Crown J, O'Leary M. The taxanes: an update. *Lancet*. 2000;355: 1176–1178.
42. Sollott SJ, Cheng L, Pauly RR, Jenkins GM, Monticone RE, Kuzuya M, Froehlich JP, Crow MT, Lakatta EG, Rowinsky EK, Kinsella JL. Taxol inhibits neointimal smooth muscle cell accumulation after angioplasty in the rat. *J Clin Invest*. 1995;95:1869–1876.
43. Wang TH, Wang HS, Ichijo H, Giannakakou P, Foster JS, Fojo T, Wimalasena J. Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. *J Biol Chem*. 1998;273:4928–4936.
44. Stahli BE, Camici GG, Steffel J, Akhmedov A, Shojaati K, Graber M, Luscher TF, Tanner FC. Paclitaxel enhances thrombin-induced endothelial tissue factor expression via c-Jun terminal NH2 kinase activation. *Circ Res*. 2006;99:149–155.
45. Steffel J, Hermann M, Greutert H, Gay S, Luscher TF, Ruschitzka F, Tanner FC. Celecoxib decreases endothelial tissue factor expression through inhibition of c-Jun terminal NH2 kinase phosphorylation. *Circulation*. 2005;111:1685–1689.
46. Finn AV, Kolodgie FD, Harnek J, Guerrero LJ, Acampado E, Tefera K, Skorija K, Weber DK, Gold HK, Virmani R. Differential response of delayed healing and persistent inflammation at sites of overlapping sirolimus- or paclitaxel-eluting stents. *Circulation*. 2005;112:270–278.
47. Halkin A, Stone GW. Polymer-based paclitaxel-eluting stents in percutaneous coronary intervention: a review of the TAXUS trials. *J Interv Cardiol*. 2004;17:271–282.
48. Suzuki T, Kopia G, Hayashi S, Bailey LR, Llanos G, Wilensky R, Klugherz BD, Papandreou G, Narayan P, Leon MB, Yeung AC, Tio F, Tsao PS, Falotico R, Carter AJ. Stent-based delivery of sirolimus reduces neointimal formation in a porcine coronary model. *Circulation*. 2001; 104:1188–1193.
49. Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, Bruns CJ, Zuelke C, Farkas S, Anthuber M, Jauch KW, Geissler EK. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. *Nat Med*. 2002;8:128–135.
50. Matter CM, Rozenberg I, Jaschko A, Greutert H, Kruz DJ, Wnendt S, Kuttler B, Joch H, Grunenfelder J, Zund G, Tanner FC, Luscher TF. Effects of tacrolimus or sirolimus on proliferation of vascular smooth muscle and endothelial cells. *J Cardiovasc Pharmacol*. 2006;48: 286–292.
51. Parry TJ, Brosius R, Thyagarajan R, Carter D, Argentieri D, Falotico R, Siekierka J. Drug-eluting stents: sirolimus and paclitaxel differentially affect cultured cells and injured arteries. *Eur J Pharmacol*. 2005;524: 19–29.
52. Guagliumi G, Farb A, Musumeci G, Valsecchi O, Tespili M, Motta T, Virmani R. Images in cardiovascular medicine: sirolimus-eluting stent implanted in human coronary artery for 16 months: pathological findings. *Circulation*. 2003;107:1340–1341.
53. Urao N, Okigaki M, Yamada H, Aadachi Y, Matsuno K, Matsui A, Matsunaga S, Tateishi K, Nomura T, Takahashi T, Tatsumi T, Matsubara

H. Erythropoietin-mobilized endothelial progenitors enhance reendothelialization via Akt-endothelial nitric oxide synthase activation and prevent neointimal hyperplasia. *Circ Res*. 2006;98:1405–1413.

54. Griese DP, Ehsan A, Melo LG, Kong D, Zhang L, Mann MJ, Pratt RE, Mulligan RC, Dzau VJ. Isolation and transplantation of autologous circulating endothelial cells into denuded vessels and prosthetic grafts: implications for cell-based vascular therapy. *Circulation*. 2003;108: 2710–2715.
55. Butzal M, Loges S, Schweizer M, Fischer U, Gehling UM, Hossfeld DK, Fiedler W. Rapamycin inhibits proliferation and differentiation of human endothelial progenitor cells in vitro. *Exp Cell Res*. 2004;300:65–71.
56. Chen TG, Chen JZ, Wang XX. Effects of rapamycin on number activity and eNOS of endothelial progenitor cells from peripheral blood. *Cell Prolif*. 2006;39:117–125.
57. Windecker S, Simon R, Lins M, Klauss V, Eberli FR, Roffi M, Pedrazzini G, Moccetti T, Wenaweser P, Togni M, Tuller D, Zbinden R, Seiler C, Mehilli J, Kastrati A, Meier B, Hess OM. Randomized comparison of a titanium-nitride-oxide-coated stent with a stainless steel stent for coronary revascularization: the TiNOX trial. *Circulation*. 2005;111: 2617–2622.
58. Aoki J, Serruys PW, van Beusekom H, Ong AT, McFadden EP, Sianos G, van der Giessen WJ, Regar E, de Feyter PJ, Davis HR, Rowland S, Kutryk MJ. Endothelial progenitor cell capture by stents coated with antibody against CD34: the HEALING-FIM (Healthy Endothelial Accelerated Lining Inhibits Neointimal Growth-First In Man) Registry. *J Am Coll Cardiol*. 2005;45:1574–1579.
59. Blindt R, Vogt F, Astafieva I, Fach C, Hristov M, Krott N, Seitz B, Kapurniotu A, Kwok C, Dewor M, Bosserhoff AK, Bernhagen J, Hanrath P, Hoffmann R, Weber C. A novel drug-eluting stent coated with an integrin-binding cyclic Arg-Gly-Asp peptide inhibits neointimal hyperplasia by recruiting endothelial progenitor cells. *J Am Coll Cardiol*. 2006;47:1786–1795.
60. Joner M, Farb A, Cheng Q, Finn AV, Acampado E, Burke AP, Skorija K, Creighton W, Kolodgie FD, Gold HK, Virmani R Pioglitazone inhibits in-stent restenosis in atherosclerotic rabbits by targeting transforming growth factor-β and MCP-1. *Arterioscler Thromb Vasc Biol*. 2007;27: 182–189.
61. Camici GG, Steffel J, Akhmedov A, Schafer N, Baldinger J, Schulz U, Shojaati K, Matter CM, Yang Z, Luscher TF, Tanner FC. Dimethyl sulfoxide inhibits tissue factor expression, thrombus formation, and vascular smooth muscle cell activation: a potential treatment strategy for drug-eluting stents. *Circulation*. 2006;114:1512–1521.
62. US Food and Drug Administration. Update to FDA statement on coronary drug-eluting stents. Available at: http://www.fda.gov/cdrh/news/010407.html. Accessed February 10, 2007.